University of Alberta

UNC-45 in the *Caenorhabditis elegans* germline and early embryo.

by

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Abstract

Myosin molecules represent a major class of motors present in all eukaryotic cells that are necessary for muscle contraction, cell division and many other processes. Obtaining the correct three dimensional structure of the head domain must occur during protein folding in order for myosin to bind actin and contract. Chaperones are a class of proteins that facilitate proper protein folding by interacting with newly synthesized proteins or misfolded proteins. UNC-45 is a molecular co-chaperone that acts at the myosin head domain to ensure the proper folding of the actin binding domain. There is one UNC-45 gene in *Caenorhabditis elegans* and it has been shown to physically interact with type II myosins in the body wall muscle. Previous results also suggested that UNC-45 was required as a maternally contributed protein. In this thesis, I have characterized the function of maternal UNC-45 in the early embryo during cytokinesis, polarity establishment and polar body extrusion and identified it as an essential molecule ensuring non-muscle myosin contractility. Additionally I have shown that UNC-45 is required in the germline for cellularization of both oocytes and sperm, representing the first detailed examination of the mechanics of germline cellularization. Finally, I have identified two distinct phases of myosin regulation during cellularization of oocytes suggesting complex regulatory pathways controlling the spatial and temporal distinction between the two phases of membrane growth. The identification of roles for UNC-45 with non-muscle myosins has revealed that UNC-45 is a more universal myosin chaperone than previously suggested as well as revealing that UNC-45 must function in folding of the myosin head domain and not simply as an assembly factor for the muscle sarcomere.

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Abbreviations used in this thesis

A/P – anterior/posterior
ACD – assembly competence domain
ADP – adenosine diphosphate
ATP – adenosine triphosphate
CCT – chaperonin-containing TCP 1 complex
cDNA – complementary deoxyribonucleic acid
CHIP - carboxyl-terminus of Hsp70 interacting protein
DAPI - 4'6-diamidino-2-phenylindole·2HCl
dsRNA – double stranded ribonucleic acid
FITC - fluorescein isothiocyanate
GC UNC-45 – general cell UNC-45
GEF – guanine nucleotide exchange factor
GFP – green fluorescent protein
GTPase – guanine triphosphatase
Hsp – heat shock protein
let - lethal
MHC – myosin heavy chain
MLC – myosin light chain
MLCK – myosin light chain kinase
mRNA – messenger ribonucleic acid
NMY– non-muscle myosin gene
Pat – paralyzed at two fold stage
PCR – polymerase chain reaction
PGC – primordial germ cells
PH – pleckstrin homology domain
PIPES – 1, 4 piperazinediethanesulfonic acid
RhoGAP – rho GTPase activating protein
RNAi – ribonucleic acid interference
ROCK – rho dependent kinase
SM UNC-45 – striated muscle UNC-45
TPR – tetratricopeptide repeat protein domain
ts – temperature sensitive
UCS – UNC-45/Cro-1/She4p protein domain
UNC– uncoordinated gene
UPP – ubiquitin proteasome pathway

1 Introduction

The contraction of the molecular motor myosin is required for movement in animals from yeast to humans. Myosin is used in muscles to allow for organismal movement and also works at the cellular level to allow for cellular trafficking, making myosin function essential for life in eukaryotes. One molecule responsible for ensuring that myosin is functional is its molecular chaperone, *uncoordinated 45* (UNC-45). UNC-45 was originally identified in worms as essential for muscle function and homologs have since been found in a diverse array of organisms from fungal species to fish and humans. Chaperones function by promoting the proper conformation of proteins and this conformation is essential to the correct function of any particular protein. Any errors in folding of proteins can have drastic consequences to the cell. Some diseases that are caused by loss of chaperone activity range from neurodegenerative diseases like Alzheimer's to muscle wasting diseases such as cardiomyopathies. Therefore it is essential to understand how and where chaperones function to promote proper protein folding.

This thesis will focus on the role of UNC-45 outside the muscle in the nematode *C. elegans,* and its role in promoting the contractile function of a non-muscle myosin NMY-2. UNC-45 plays a role in ensuring proper body wall muscle function in both invertebrate and vertebrate systems and there is evidence that UNC-45 in *C. elegans* also functions outside the muscle. Additionally, vertebrates may also have UNC-45 function outside the muscle because they have a defined muscle specific homolog for

UNC-45 as well as a homolog that is more ubiquitous. *C. elegans* has only one UNC-45 gene that is likely required for both muscle and non-muscle processes. No function for UNC-45 outside the body wall muscle in nematodes had been established prior to my work. However, there is some genetic evidence that UNC-45 is required outside the muscle. Identifying functions of UNC-45 outside the muscle will suggest that UNC-45 and its homologs are more universal chaperones than previously suggested and will indicate that there are conserved pathways for myosin folding between muscle and non-muscle varieties. We can use the worm as a model organism to study the role of UNC-45 in non-muscle systems in order to further characterize the role of this conserved myosin chaperone.

1.1 Structure and function of myosins

Molecular motors within the cell allow for movement of animals by muscle contraction and movement within the cell for vesicle trafficking and other cell shape changes. For instance, striated muscle is used in many organisms for locomotion and the sole contractile class of molecules in muscle is myosin. Myosin is a molecular motor that has a complex three dimensional structure that allows for its contractile ability. The three dimensional structure of myosin is critical to its function and the proper folding of the head domain, in particular, is essential for every eukaryotic cell.

There are 18 different classes of myosins found amongst all organisms (reviewed in Berg *et al.* 2001) and the main myosins present in the muscle are Type II myosins. Type II myosins are characterized by a two-headed structure with a long coil-coiled tail domain that can be both muscle and non-muscle associated (Figure 1.1). The functional myosin complex includes a myosin heavy chain dimer and two types of light chains per heavy chain: one regulatory and one essential light chain. These light chains associate with the neck region of the myosin heavy chain between the head and the coiled-coil tail, thus forming a hexameric contractile molecule. The role of the light chains during myosin regulation and contraction will be discussed in section 1.8. The difference between Type II non-muscle and muscle associated myosins is that non-muscle myosins do not associate into thick filament structures that are characteristic of muscle sarcomeres (discussed in section 1.3). Type II non-muscle myosins are required for contractile activities within the cell such as cytokinesis at the end of cell division. Type II non-muscle myosins contract during processes such as cytokinesis by forming antiparallel dimers at the extreme C terminus. This antiparallel binding is also required for nucleation of myosin assembly into thick filaments of muscle.

Myosin molecules contain two distinct domains: a complex globular head domain and a coiled-coil rod domain (Figure 1.1A). All regions of the myosin motor must be present for function and, more importantly for this thesis, properly folded for myosin function. The myosin head contains the approximately 780 residue contractile domain in the amino terminal region of the protein (Rayment *et al.* 1993). The myosin head domain of all types of myosin contains two subdomains that bind either actin or ATP. The crystal structure of the Type II myosin head has been known for some time where the core folding motif consists of seven, mostly parallel, beta pleated sheets that are flanked by three alpha helices on each side to form the ATP binding pocket (Figure 1.1B) (Winkelmann *et al.* 1991; Rayment *et al.* 1994; Rayment 1996). One feature of the folded myosin head is a deep cleft that runs between the actin and ATP binding domains that separates these two regions into different domains (Figure 1.1B). The cleft may provide flexibility to the head to allow pivoting during myosin contraction (Conibear *et al.* 2003; Ruppel and Spudich 1996). Perhaps one of the key folding events is the distinction between the ATP pocket and the actin binding domain to form the cleft.

Myosin is a microfilament associated molecular motor that contracts as a result of its ATPase activity. The hydrolysis of ATP in the head domain of myosin can change conformation to result in the movement of myosin along actin tracts. Upon ATP binding the myosin releases from the actin subunits (Figure 1.2A); the subsequent hydrolysis of ATP causes a conformation change in the myosin that allows it to associate with actin (Figure 1.2B and C). ADP then releases from the myosin causing the myosin head to contract until ATP once again binds myosin to release it off the actin (Figure 1.2D). Myosin may propel cargo along the actin filaments by tethering to vesicles and other cargo at the tail region. Despite the knowledge of the crystal structure of the myosin head and the details of contraction surprisingly little is known about the folding kinetics of the different regions of the myosin head domain.

1.1.1 Type II myosin folding

Myosin cannot be easily folded in *in vitro* systems, likely because muscle specific chaperones are needed to fold the complex myosin head (Barral *et al.* 2002; Srikakulam and Winkelmann 1999; Hutagalung *et al.* 2002; Chow *et al.* 2002). Chaperones such as heat shock protein 90 and 70 (Hsp90 and Hsp70 respectively) complex with all newly synthesized myosin molecules that contain a partially folded motor domain (Srikakulam and Winkelmann 1999). However, Hsp70 and Hsp90 are ubiquitous chaperones present in all tissues suggesting that specificity factors must be involved in folding the muscle myosin head *in vivo.* Incompletely folded myosin molecules remain associated with the folding chaperones but can complete light chain association and tail dimerization prior to completion of head folding (Figure 1.3A and B) (Chow *et al.* 2002). Head folding is the rate limiting step of myosin formation and loosely organized filaments remain associated with chaperones such as Hsp90; possibly to prevent release of rogue motors into the cytoplasm until the thick filament is fully formed (Srikakulam and Winkelmann 2004). Myosin chaperones may not only facilitate head folding but also prevent myosin contraction by blocking actin binding until the head is fully folded. Filaments form because the tail domain can spontaneously self-assemble into two-headed dimers although the initiation of dimerization may require the chaperonin CCT (Figure 1.3B) (Srikakulam and Winkelmann 1999). Chaperonins, like CCT, are a class of chaperones that consist of a multisubunit ring structure that likely provide a sequestered environment that favors folding (Valpuesta *et al.* 2002). These short filamentous structure with incompletely folded myosin head domains represent an intermediate in the myosin folding and assembly pathway (Figure 1.3C and D) (Srikakulam and Winkelmann 2004).

Most of the research done on myosin head folding has used striated muscle myosins, and although it has not been directly examined, type II non-muscle myosins likely have a similar folding pathway to muscle myosins but they but do not undergo the final step of assembly into striated filaments. Only two processes must occur for non-muscle myosin to attain a functional conformation: 1) folding of the myosin head domain and 2) assembly into bipolar complexes. Because of the close similarity of the dimerization domains between muscle and non-muscle myosins it is likely that dimerization of non-muscle myosins also occurs without the aid of specific chaperones. Identification of myosin specific chaperones becomes important to understand not only the folding cascade of myosin but also protein conformation diseases.

1.1.2 Type II myosin assembly

Muscle myosins assemble into a complicated sarcomere and this assembly process may also require chaperones. There are three steps to myosin assembly that must occur prior to filament function. The first is dimerization of the myosin coiled-coil tails (Chow *et al.* 2002; Barral and Epstein 1999). The second assembly step is the formation of tail-tail antiparallel myosin dimers. This step is also spontaneous and requires the assembly competence domain (Barral and Epstein 1999). Finally, the assembly into the sarcomeric thick filament of all the myosin molecules is an intrinsic property of myosin and can occur spontaneously but may require other factors (Figure 1.3E). Processes such as regulation of sarcomeric length as well as other species specific properties of thick filament assembly require molecules such as titin (reviewed in Trinick 1996) and paramyosin (reviewed in Epstein 1990) to name two. The final assembly stage recruits many different factors into the final sarcomere and is likely to involve many different chaperone or chaperone-like molecules to facilitate sarcomeric assembly.

1.2 Chaperone function - Heat shock proteins and Cdc37

Chaperones are proteins that aid in the proper folding of nascent proteins, promote correct folding of misfolded proteins and target terminally misfolded proteins for degradation. Chaperones act by recognizing stretches of hydrophobic amino acids that are exposed upon translation or misfolding and can initiate one of three pathways: promoting proper folding by an ATP dependent mechanism, preventing aggregation by shielding the hydrophobic domains or targeting misfolded proteins for degradation by recruiting ubiquitinating enzymes and subsequent degradation by the 26S proteasome (Figure 1.4). There are many different chaperones within any given cell type and there are likely many cell-specific and even organelle specific chaperones (reviewed in Young *et al.* 2003).

1.2.1 Role of the heat shock proteins

The heat shock family of proteins was originally identified as proteins upregulated in response to thermal stress but is now understood to be essential components of the chaperone network in all cells. One of the members of the heat shock family has been implicated with UNC-45 mediated chaperone activity is heat shock protein 90 (Hsp90).

Hsp90 has chaperone activity on a wide variety of client proteins and acts later in the folding cascade than another heat shock protein chaperone, Hsp70. The folding cascade begins during translation where many proteins require assistance achieving their final conformation. Hsp70 is a chaperone that acts in a large multisubunit complex responsible for interacting with hydrophobic stretches of amino acids in nascent polypeptides to prevent the aggregation of incompletely translated peptides and is also required for folding the new protein. Hsp70 chaperone activity typically assists in folding by associating with exposed hydrophobic surfaces using several rounds of ATP dependent substrate binding and release. When the degree of unfolding is terminal, the Hsp70 complex can interact with CHIP (carboxyl-terminus of Hsp70 interacting protein) and other proteins that ubiquitinate the peptide to target it for degradation by the ubiquitin proteasome pathway (UPP).

Comparatively little is known about the molecular function of Hsp90 but it is known that there is a diverse array of co-chaperones that provide specificity to Hsp90 to act on an even more diverse set of client proteins. Hsp90 acts as a large multisubunit chaperone complex that also contains Hsp70 and other co-chaperones but may arrive later to the complex to facilitate completion of folding (reviewed in Kimmins and MacRae 2000). Heat shock protein 90 functions to facilitate interaction of the client protein with a ligand (Young *et al.* 2003; Nathan *et al.* 1997; reviewed in Pearl and Prodromou 2006).

The molecular structure of Hsp90 has recently been determined from domain analysis (reviewed in Pearl and Prodromou 2006). Hsp90 forms a homodimer where the C-terminus forms a constitutive dimerization domain (Nemoto *et al.* 1995) while the N-terminus can homodimerize and this interaction is thought to facilitate ATP hydrolysis that is required for Hsp90-mediated protein folding (Figure 1.5A)(Young *et al.* 1997;

Prodromou *et al.* 2000). ATP hydrolysis promotes a conformational change in the substrate and eventual release of the correctly folded protein (Figure 1.5B). The C-terminus of Hsp90 contains a loosely conserved MEEVD domain (Figure 1.5A) that is required for interactions with tetratricopeptide repeat (TPR) domain-containing co-chaperone proteins including UNC-45 (Young *et al.* 1998; Owens-Grillo *et al.* 1996; Barral *et al.* 2002).

Hsp90 alone is unable to catalyze any substantial folding changes in target proteins and requires association with co-chaperones that contribute to target specificity and folding (reviewed in Pearl and Prodromou 2006). The Hsp90-co-chaperone complex is likely required to induce subtle changes in the folding or arrangement of domains in substantially folded client proteins. This chaperone activity of Hsp90 is likely insufficient for a fully folded client protein and often requires specificity factors such as cyclophilin 40 (Carrello *et al.* 2004) as well as co-chaperones that have endogenous chaperone activity such as UNC-45 and Cdc37. However, the detailed molecular mechanism of this co-chaperone activity is unknown.

1.2.2 Heat shock protein co-chaperones – UNC-45 and Cdc37

Although Hsp90 has chaperone activity, often it is the associated co-chaperone that contributes to the folding cascade and also provides specificity. Cdc37 is a co-chaperone that facilitates folding of protein kinases in many systems and is similar to UNC-45 because it also has endogenous chaperone activity (reviewed in MacLean and Picard 2003). Cdc37 acts as an Hsp90 co-chaperone to direct Hsp90 to a more limited set of client proteins. Based on the functional similarity between Cdc37 and UNC-45, we can suggest potential biochemical functions of UNC-45 based on data collected for Cdc37.

Cdc37 is a protein in budding yeast that directly interacts with both client proteins and Hsp90. Cdc37 acts as a co-chaperone by inhibiting the ATPase activity of Hsp90 that may allow for a longer and stronger interaction between Hsp90 and its client (Siligardi *et al.* 2002). The association of Cdc37 and Hsp90 is strengthened by the addition of the client protein suggesting that the ternary complex of Hsp90/Cdc37/client is most stable (Shao *et al.* 2001). The actual function of Cdc37 is unclear but there are three possibilities: Cdc37 could 1) stabilize unfolded or misfolded clients by recruiting Hsp90; 2) enhance the Hsp90/client protein interaction or 3) directly act on the client as a chaperone. Although Cdc37 and UNC-45 do not share similarity at the protein level outside the Hsp90 binding domain, the possible mechanisms of Cdc37 chaperone activity are also applicable to UNC-45 in its possible roles for catalyzing myosin function. However, UNC-45 is required to chaperone a multimeric structure of the sarcomere thus adding another possible function for UNC-45 where it promotes myosin dimer assembly into the crystalline sarcomeric array.

1.3 History of UNC-45 in the worm

In *C. elegans* the 95 body wall muscle cells are organized into four quadrants lining the body cavity and attached to the hypodermis (Figure 1.6A and B) (Riddle *et al.* 1997). Muscle tissue, including body wall muscle of nematodes and skeletal muscle of vertebrates, is composed of an organized set of muscle cells. Within each muscle cell in the worm and vertebrates there are highly organized contractile structures called sarcomeres. The 10 µm sarcomeres are comprised of thick filaments and thin filaments where thick filaments are bipolar tubular assemblies formed by myosin and thin filaments are filamentous actin polymers (Figure 1.6D) (Waterston 1988). Thick filaments form part of the sarcomere, and are composed of a near crystalline array of myosin molecules.

Mutants in muscle function and assembly were found in a temperature sensitive screen for movement defective animals (Epstein and Thomson 1974; Venolia and Waterston 1990). A mutation in *unc-45* allowed normal movement of the adult worm at the permissive temperature (15°C) but when transferred to the restrictive temperature (26°C) the mutants exhibited complete paralysis. (The nomenclature convention in *C. elegans* has *unc-45* referring to the gene and UNC-45 referring to the protein). Paralysis was the result of severely disorganized body wall muscles; specifically the organization of the myosin molecules in the muscle (Figure 1.6E and F) (Barral *et al.* 1998). Because *unc-45* mutants show disorganized and reduced myosin-containing thick filaments it was hypothesized that UNC-45 is involved in assembling or maintaining the body wall muscle sarcomeres (Epstein and Thomson 1974; Venolia and Waterston 1990). Close observation of the temperature sensitive allele (e286) revealed that adult animals transferred to the restrictive temperature after the completion of body wall muscle formation (as adults) did not exhibit the unc phenotype. However, younger worms (L4 larvae) undergoing muscle development transferred to the restrictive temperature were paralyzed (Barral *et al.* 1998). These observations lead researchers to propose that *unc-45* most likely acts to aid in body wall muscle assembly because assembly occurs mostly during the L4 larval-adult transition.

1.3.1 Type II muscle myosin folding and assembly in *C. elegans*

Nematode thick filaments are composed of two different myosin heavy chains, A and B (Schachat *et al.* 1977). Myosin heavy chains A and B are type II class of myosins that possess coiled-coil tails that allow for dimerization and mediate assembly into thick filaments of muscle (reviewed by Berg *et al.* 2001). The myosin heavy chains (MHC) are localized to different regions of the thick filament in *C. elegans*; MHC A/*myo*-3 is restricted to the central 2 μm region of the sarcomere whereas MHC B/*unc-54* is present along the majority of the sarcomere but is excluded from the cent[illegible]region (Miller *et al.* 1983). The biochemical explanation for this localization pattern is that MHC A contains a non-helical tailpiece that promotes antiparallel dimerization in order to seed or nucleate filament formation. MHC B does not have the ability to form tail-tail dimers and is excluded from the midregion of the thick filament.

UNC-45 was shown to localize specifically with MHC B and not MHC A in the thick filaments, implying a specific role with MHC B as opposed to MHC A (Figure 1.7) (Ao and Pilgrim 2000). Additionally, UNC-45 protein fails to localize properly in muscle lacking MHC B, for instance, in MHC B/*unc-54* null mutant backgrounds (Ao and Pilgrim 2000). These results suggest that UNC-45 may play a role as an assembler for thick filament formation specifically with loading of MHC B onto the nascent thick filament nucleated by MHC A tail-tail dimerization.

1.3.2 Proposed mechanisms of UNC-45 chaperone activity

From the genetic studies of UNC-45 mutants two possible mechanisms were proposed that are not mutually exclusive: 1) UNC-45 acts as a myosin chaperone to aid in the folding of the complex myosin head (Figure 1.8A and C) or 2) UNC-45 acts as an assemblase aiding in the recruitment and organization of MHC B into the thick filaments (Figure 1.8B).

UNC-45 may function as an assemblase but because UNC-45 acts with non-sarcomeric myosins it also likely functions as a chaperone for the initial folding of the myosin head domain (Figure 1.8A). Additionally, it is known that UNC-45 remains associated with fully functional, and folded, myosin in the body wall muscle (Ao and Pilgrim 2000) suggesting that another function of UNC-45 is required after initial folding of the myosin. The C-terminal Unc-45/CRO1/She4p-like (UCS) domain can interact with the neck region of type II muscle myosins (Horlick 2005) but the neck region is unlikely to require chaperone activity in folding (Srikakulam and Winkelmann 1999) therefore its localization is unexpected. At least in the muscle, UNC-45 may remain closely associated with the myosin head by being stored at the neck region in order to restore myosin function during contraction induced misfolding of the myosin head (Figure 1.8). Alternatively, the UCS domain could simply be acting as a recognition site for myosin and allow for Hsp90 co-chaperone specificity to recruit Hsp90 to the myosin head. This latter possibility suggests that UNC-45 has no chaperone activity at the myosin head but only acts as a specificity factor for Hsp90. This possibility is also unlikely because the UCS domain is required for the chaperone activity but the TPR domain recruits Hsp90.

Because the initiation of thick filament formation and myosin assembly is mostly spontaneous *in vitro* and does not require additional factors, it is hypothesized that UNC-45 is not required for myosin assembly and is likely only required for the rate limiting step of myosin head folding. It remains possible that UNC-45 is required for thick filament assembly but I will show in this thesis UNC-45 also acts on myosins that do not form filaments. Therefore UNC-45 may still be required for thick filament assembly in the muscle but it has additional roles in non-muscle myosin folding and/or assembly as well.

1.3.3 Evidence for UNC-45 function outside the muscle in nematodes

Temperature-sensitive alleles have given us insight into the role of UNC-45 in the adult body wall muscle but the alleles used did not completely abolish function. Lethal alleles result in a two-fold arrested embryo called the 'Pat' (paralyzed at two-fold) phenotype characterized by failed hatching, loss of pharyngeal pumping and movement within the eggshell (Venolia and Waterston 1990). Similarly, other mutants in body wall components also exhibit two-fold arrest, for instance mutants in *myo-3* (myosin heavy chain A (MHC A)) show the Pat phenotype (Williams and Waterston 1994); however, it remains unclear whether the Pat phenotypes of *unc-45* and *myo-3* mutants are related. Pharyngeal pumping is not observed in these pat embryos suggesting that UNC-45 may also be responsible for coordinating the function of pharyngeal myosins and indeed there are two Type II myosins required in the pharynx (MHC C and MHC D) (Miller *et al.* 1986).

These observations suggested that UNC-45 is required outside the body wall muscle or that UNC-45 functions with another myosin in addition to MHC B. The predicted chaperone activity of UNC-45 in the muscle provided a hypothesis to investigate the function of UNC-45 outside the adult body wall muscle and will be the focus of this thesis.

1.3.4 Structure and function of UNC-45

UNC-45::GFP transgenic worms show expression of UNC-45 in the muscles of the body wall, vulva, anal depressor and pharynx (Ao and Pilgrim 2000). UNC-45::GFP as well as antibody staining revealed that it co-localizes with a myosin heavy chain in the body wall muscle, MHC B (Ao and Pilgrim 2000; Horlick 2005). Analysis of the structure of the UNC-45 protein has revealed potential chaperone domains based on sequence similarities to known proteins from other systems. The 961 amino acid UNC-45 protein product contains three domains: a tetratricopeptide (TPR) domain, unique central domain and a CRO1/She4p/Unc-45-like domain (UCS) (Figure 1.9).

Three TPR repeats are present at the N-terminus of UNC-45 (Venolia *et al.* 1999) and are domains involved in protein-protein interactions (reviewed by Blatch and Lassle 1999). TPR domains are loosely conserved 34-amino acid repeats that were originally discovered as a domain in the anaphase promoting complex (Sikorski *et al.* 1990) and have since been found in a diverse range of proteins. The TPR domain of UNC-45 binds Hsp90, suggesting that Hsp90 and UNC-45 may act as co-chaperones for myosin assembly (Barral *et al.* 2002). Hsp90 and UNC-45 interact directly by co-immunoprecipitation and this interaction requires the TPR domain of UNC-45 (Barral *et al.* 2002). Hsp90 tends to function with partially folded intermediates as opposed to chaperoning the initial folding of a polypeptide (Brown, M.A. *et al.* 2007) therefore it can be hypothesized that UNC-45 acts with Hsp90 late in the myosin head folding cascade to promote the folding of an intermediate into the eventual fully folded conformation. Hsp90 in different systems has been found to complex with UNC-45 homologs including Rng3p in fission yeast (Mishra *et al.* 2005) and Unc45b in zebrafish (Etard *et al.* 2007) (see section 1.4). The fungal UCS homologs do not contain a TPR domain suggesting that the UCS/Hsp90 interaction in these systems occurs through a separate region of the protein.

BLAST analysis has not revealed any statistically similar sequences in the database to the central domain of UNC-45 and its function remains unknown. The hypothesized function of this domain is to act as a non-specific linker region that allows for the TPR and UCS domains to interact with different regions of target proteins (Venolia *et al.* 1999). However, this hypothesis remains to be confirmed and is not the focus of this thesis. The C-terminus is required to bind the myosin head domain and for the stability of thick filaments in body wall muscle (Barral *et al.* 1998; Barral *et al.* 2002). Of the *unc-45* mutant alleles known, the most severe lethal alleles are those that result in a premature stop codon that removes the UCS domain (Figure 1.9). However, the most informative alleles have been the temperature-sensitive alleles where three of the four alleles contain missense mutations in the UCS domain indicating that the UCS domain is essential for UNC-45 function (Figure 1.9) (Barral *et al.* 1998). UNC-45 alone has endogenous chaperone activity where it can prevent thermal aggregation of citrate synthase as well as with scallop myosin subfragment 1 (Barral *et al.* 2002). And this chaperone activity occurs whether the TPR domain is present or not (Barral *et al.* 2002). Therefore Hsp90 is not acting as the sole chaperone in the UNC-45/Hsp90 complex. Additionally, data from fungal models show that the UCS domain is necessary and sufficient to promote myosin function indicating that the C-terminal portion of the protein contains the catalytic domain (Lord and Pollard 2004) (discussed in section 1.4.1). Therefore it is likely that the UCS domain is required to promote proper myosin folding and/or assembly.

1.4 Chaperones in other invertebrate systems

The fungal systems that have UCS homologs do not have sarcomeres, indicating that the UNC-45 family members likely have roles with non-muscle myosins, and looking at these systems may suggest some additional functions for UNC-45 outside the muscle. The UCS domain shows similarity with at least three studied proteins: CRO1 is involved in the developmental switch from syncytial to cellular fates in a filamentous fungus, *Podospora anserina,* (Berteaux-Lecellier *et al.* 1998); Rng3 in *Schizosaccharomyces pombe* functions in myosin assembly in the contractile ring for cytokinesis (Wong *et al.* 2000); and She4p, which is involved in segregating Ash1p to the daughter cell of the budding yeast, *Saccharomyces cerevisiae* (Table 1.1; Figure 1.9B) (Jansen *et al.* 1996; Bobola *et al.* 1996). Therefore, this domain is involved in pathways functioning in intracellular movement of other organisms and may carry out similar functions in *C. elegans*.

1.4.1 Rng3 in *Schizosaccharomyces pombe*

One of the members of the UCS family of proteins is Rng3p, a fission yeast homologue that shares a C-terminal domain with UNC-45. Rng3p was identified as a crucial component of assembly of the cytokinetic actomyosin ring in *Schizosaccharomyces pombe* (Wong *et al.* 2000). Rng3p was shown to genetically interact with a type II myosin component of the actomyosin ring, *myo2*. Single and double mutants of the *rng3-65* mutant allele and the *myo2-E1* allele resulted in improper assembly of the actomyosin ring and the phenotype of the double mutant is not enhanced leading the authors to conclude that Rng3p and Myo2p genetically interact (Wong *et al.* 2000). In *myo2–E1* mutants, Rng3p::GFP localized to a spot near the division plane prior to actomyosin assembly and this localization was not observed in wildtype (Wong *et al.* 2002). This localization pattern was unique to only one of the three *myo2* alleles tested (*myo2-E1*) that contained a mutation in the contractile head domain of the myosin. The conclusion was that Rng3p normally functions to fold the myosin head before myosin associates with the contractile ring. When Rng3p cannot complete folding of Myo2p due to a mutation in the myosin head, Rng3p remains associated with myosin; thus localizing to the myosin spot prior to ring assembly. The authors suggested that Rng3p may be required to maintain Myo2p in a state competent for actomyosin ring assembly possibly in a similar mechanism to other UCS homologs (reviewed in Hou and McCollum 2002).

Alternative data suggests that Rng3p does indeed localize to the actomyosin ring in wildtype (Lord and Pollard 2004). The Pollard group used triple GFP or YFP tagged Rng3p to increase the strength of the signal and found that Rng3p associates with the actomyosin ring after Myo2p localization. These results are in direct contradiction to those found by Wong *et al.* (2000) where they suggested that Rng3p functions prior to Myo2p localization and activity at the cortex. Although this debate has not been resolved, both groups have drawn the same conclusion that Rng3p functions as a myosin chaperone to ensure the functioning of the contractile head domain. There is possibly a cytosolic association where UNC-45 related chaperones associate with myosin to begin folding the head domain and a second function at the site of action, either muscle or cortex, to assemble the myosin into contractile structures. Rng3p can restore Myo2p folding *in vitro* (Wong *et al.* 2000) and is necessary and sufficient to restore Myo2p function in a gliding filament assay (Lord and Pollard 2004). More precisely, Lord and Pollard (2004) found that only the C-terminal UCS domain of Rng3p was sufficient to restore purified Myo2p activity suggesting that the UCS domain in other homologs has a conserved function in myosin folding.

There are clear genetic interactions between *rng-3* and *myo-2* and requirements for each other *in vitro;* one piece of the puzzle that was missing was a physical interaction between the myosin and its chaperone. Mishra *et al.* (2004) found that Rng3p and Myo2p interact and they also found that the Hsp90 homolog, Swo1p, complexes with Rng3p/Myo2p. Taken together these results suggest that an Hsp90 homolog is required for co-chaperone function similar to the UCS protein function in other systems (Barral *et al.* 2002).

S. pombe has provided a simple system to perform biochemistry on the UCS-containing proteins that has revealed conserved roles between fungal and nematode homologs. Fission yeast is a wonderful model to study cytokinesis and many proteins show functional conservation between yeast and higher eukaryotes. Therefore much can be learned about the potential role of UNC-45 in embryonic cytokinesis by looking at its counterpart in fission yeast.

1.4.2 Cro1 in *Podospora anserina*

Podospora anserina is a filamentous fungus that undergoes both syncytial and cellular life stages. The transition between syncytial and cellular life is analogous to germline cellularization in the worm and components of these processes may be conserved. The primary defect in mutants for *cro1* is an inability to cellularize resulting in croziers with abnormal number of nuclei from the normal two to up to twelve (Berteaux-Lecellier *et al.* 1998). The role of *cro1* in *P. anserina* suggests a possible function for UNC-45 in the germline of *C. elegans* because the germline undergoes a switch from a syncytial state in the gonad and eventually gets cellularized into individual germ cells. Therefore, CRO1 and UNC-45 may have mechanistic similarities in their roles on individual myosins or other potential client proteins.

1.4.3 She4p in *Saccharomyces cerevisiae*

She4p is the UCS homolog found in budding yeast (*Saccharomyces cerevisiae)* (Jansen *et al.* 1996; Wendland *et al.* 1996). The C-terminus of the protein contains a UCS domain with 50% similarity to the Cro1 homolog and 43% similarity to its homolog in fission yeast. Mutants in She4p affect the asymmetry of mating type switching, organization of the microfilament cortex and have a mild cytokinesis defect. The phenotypes observed were similar to that of knockdowns of both class I and V myosins and yeast-two hybrid assays and co-immunoprecipitation experiments completed by the Jansen group (Wesche *et al.* 2003) found that She4p interacts with Class I myosins (Myo3p/Myo5p) and Class V myosins (Myo2p/Myo4p). More specifically the She4p

UCS domain interacts with the myosin head domain of Class I myosins in a region correlating to the actin binding domain (Wesche *et al.* 2003; Toi *et al.* 2003). Similar to what was observed for Rng3p in *S. pombe,* Wesche *et al.* (2003) found that the UCS domain was necessary and sufficient for myosin interaction. She4p was not found to interact with Class II myosins suggesting that She4p in budding yeast plays a different role than its UCS homologs in other systems. These results indicate that UCS homologs may be general myosin chaperones and their function is not restricted to Class II myosins.

1.5 Vertebrate UNC-45 homologs

Fungal homologs of nematode UNC-45 have provided important insights into the molecular function of this conserved myosin chaperone. Analyses of other systems including fish and humans have provided additional information about the diverse roles of UNC-45. All vertebrate homologs share similar TPR and UCS domains and the TPR domain binds Hsp90 at least in fish and mouse systems (Price *et al.* 2002; Etard *et al.* 2007). In vertebrates there are two homologs of UNC-45; one is expressed ubiquitously called UNC45a. The other homolog (UNC45b) is expressed at high levels in the striated muscle suggesting that there are two distinct functions for UNC-45: universal cellular maintenance and specifically in muscle myosin function. Much of the work in fish has been on the muscle homolog where morpholino knockdowns of UNC45b show skeletal and cardiac muscle abnormalities (Wohlgemuth *et al.* 2007).

In mice, UNC-45 also has two homologs; one muscle specific and the other more ubiquitously expressed (Price *et al.* 2002). The UNC-45a homolog in mice is expressed throughout the embryo and knockdown of this homolog by antisense treatment resulted in suppressed cell proliferation. These results may indicate a role for UNC-45a in cytokinesis in vertebrate systems similar to its nematode counterpart. Humans also contain both an A and a B homolog of UNC-45. The UNC-45a homolog is overexpressed in ovarian cancers suggesting a further link between UNC-45 and cell proliferation (Bazzaro *et al.* 2007). The Bazzaro paper (2007) showed that UNC-45a localizes to the cleavage furrow with myosin II suggesting that the transformation phenotype observed is likely due to an UNC-45/myosin II association. The UNC-45a homolog is also required for chaperoning the progesterone receptor as an Hsp90 co-chaperone (Chadli *et al.* 2006). This introduces the possibility that UNC-45 has client proteins outside the myosin superfamily.

If both the general cell and striated muscle homologs of UNC-45 are chaperones for myosin and possibly other molecules, then perhaps the single worm homolog carries out all the functions of both vertebrate homologs. Therefore studying the role of UNC-45 outside the body wall muscle in *C. elegans* provides a simple system to look at potential roles for the UNC-45b homolog in vertebrates.

1.6 *C. elegans* as a model for studying UNC-45 function

The nematode, *Caenorhabditis elegans*, is an excellent model for genetic research because of its ease of genetic manipulation and its hermaphrodite lifecycle (Brenner 1974). Advances such as the discovery of RNA interference (Fire *et al.*1998) and the sequencing of the *C. elegans* genome have greatly increased our ability to use this organism for genetic analysis. The worm is transparent throughout all stages and allow for simple visualization of developmental processes. Additionally, the newly fertilized embryo is an unusually large cell and can be viewed in real time by both DIC and fluorescence microscopy.

C. elegans has a stereotyped developmental program where each cell division has determined fates resulting in a lineage of specific cells (Sulston *et al.* 1983). As a result, one can study the progenitors of any cell type independent of environment, creating an organism that can be manipulated and specific developmental lineages can be followed with precision. These stereotyped cell divisions make worms a simple system to study fate decisions at the molecular level during development.

The nematode embryo also provides a system to look at the biology of a single cell by looking at the first stages after fertilization allowing for dynamic processes to be studied in real time. Additionally, the *C. elegans* germline has been used extensively to study the regulation of the cell cycle because the developing germ cells show spatial and temporal regulation through the linear gonad of the worm (reviewed in Hubbard and Greenstein 2000). Both embryogenesis and germ cell formation require myosin motors making both of these developmental processes useful to study the function of the myosin chaperone UNC-45.

Additionally, *C. elegans* contains only one UNC-45 homolog in contrast to its vertebrate counterparts. Therefore studying the roles of a single UNC-45 gene may ultimately lead to ideas for the function of UCS family members, including human homologs.

1.7 Maternal contribution of non-muscle myosin (NMY-2) and UNC-45

Pronuclear migration, ruffling, polar body extrusion and cytokinesis are all easily identifiable processes that occur in the one-cell embryo. All of these events have a requirement for maternally provided non-muscle myosin 2 (NMY-2), a type II myosin present throughout the early embryo and germline (Guo and Kemphues 1996). Despite the many functions of NMY-2 in the early embryo, zygotic transcription in the worm does not turn on until much later in development. Thus the NMY-2 that is required in the early embryo must be maternally provided. In fact, both UNC-45 and NMY-2 mRNAs are present in large quantities throughout the hermaphrodite gonad (Figure 1.10) (Dr. Y. Kohara, Search *"unc-45"* at http://nematode.lab.nig.ac.jp /db2/KeysrchForm.php).

Genetic studies also suggest that UNC-45 must be maternally provided and has a function independent of *myo-3*. UNC-45 is required in the body wall muscle to chaperone MHC B/*unc-54*, but the *unc-45* null phenotype is more severe than the *unc-54* null phenotype. *unc-45* lethal homozygotes arrest at the two-fold stage of embryogenesis whereas MHC B null homozygotes are fertile and viable but paralyzed as adults (Epstein and Thomson 1974). The genetic evidence suggests that UNC-45 likely has an MHC B independent function. *unc-45* is known to be required maternally as shown through genetic characterization of a lethal allele, *st604*. The *st604* allele is capable of maternal rescue that is dependent upon the genotype of the mother (Venolia and Waterston 1990). Heterozygous hermaphrodites were able to produce viable and fertile, but *unc*, homozygous (*let/let*) progeny that have wild-type movement, indicating that the maternally contributed protein is sufficient to allow proper muscle formation. It was unlikely that maternally provided UNC-45 was solely required for allowing growth after two-fold stage because zygotic transcription would be sufficient at this developmental time point. Therefore maternally provided UNC-45 likely had a role in the early embryo prior to the onset of zygotic transcription in a role that would not have been discovered by traditional mutation analysis.

1.7.1 Role of maternally provided Type II myosins in the embryo

UNC-45 is required for myosin function in the body wall muscle and this function may be maintained in its maternal role. There are many potential myosin-dependent processes that maternal UNC-45 could be involved in, including cytokinesis and polarity establishment.

1.7.1.1 Cytokinesis

At the end of the mitotic cell cycle the division of genetic components must be accompanied by the physical separation of cytoplasm into daughter cells. Cytokinesis is the process of pinching off two daughter cells using an actomyosin based contractile apparatus. The basic steps of cytokinesis are similar amongst metazoans and the factors involved are also often highly conserved. One of the most conserved features of cytokinesis is the use of non-muscle type II myosins as the motor molecule for cytokinetic furrow ingression (reviewed in Glotzer 2001). Myosin II provides the major force production for furrow ingression but additional force generating factors are required in some systems (reviewed in Robinson and Spudich 2004).

At anaphase of the cell cycle, signals from the central microtubule spindle position the cleavage furrow to bisect the mitotic spindle (reviewed in Glotzer 2004). Once the complex cell signaling has occurred to position the mitotic spindle, myosin II accumulates at the future plane of cell division. Little is known about the recruitment of myosin to the cleavage furrow but it appears that different organisms have different strategies of myosin recruitment that do not require a myosin-actin interaction (Maddox *et al.* 2007; Liu *et al.* 2008; Zhou *et al.* 2008). Once assembled the actin and myosin network forms a filamentous ring around the division plane called the contractile ring (Figure 1.11). The movement of myosin along the filamentous actin reduces the diameter of the contractile ring thus resulting in separation of daughter cells. The "purse-string" model of cytokinesis (reviewed in Uyeda and Nagasaki 2004) is regulated by phosphorylation events that control myosin activity in order to time cytokinesis with mitotic events (discussed in section 1.8).

Non-muscle myosin II is the major force generating molecule in cytokinesis in worms (Glotzer 2001). Consistent with this, *nmy-2(RNAi)* knockdown in *C. elegans* embryos completely abolishes cytokinesis (Guo and Kemphues 1996). Loss of additional myosin regulatory factors also prevents furrow ingression, including the regulatory myosin light chain (Shelton *et al.* 1999) and myosin targeted kinases (Piekny and Mains 2002). Knockdown of NMY-2 or its associated light chain is sufficient to inhibit cytokinesis suggesting that myosin II is the major contractile motor protein in this process. The requirement for NMY-2 also suggests that cytokinesis may also require the myosin-associated chaperone UNC-45.

1.7.1.2 Polarity

During fertilization the sperm enters the egg at a pole of the embryo opposite the oocyte pronucleus and it is the location of sperm entry that dictates the posterior pole of the egg (Figure 1.12A). At fertilization the microfilament network interprets the posterior polarity cue from the sperm donated microtubule-organizing center and is regionally destabilized in the posterior (Figure 1.12B) (Goldstein and Hird 1996; O'Connell *et al.* 2000; Wallenfang and Seydoux 2000; reviewed in Cowan and Hyman 2007). The polarity cue was found to be CYK-4, a Rho-GTP activating protein that modulates actomyosin activity in the posterior through RhoA and ECT-2 (Jenkins *et al.* 2006). Upon sperm entry the microfilament cortex redistributes to concentrate into an anterior domain. As the microfilament cortex retracts, it pulls its associated proteins to the anterior end as well, specifically the PAR-3/PAR-6/PKC-3 complex (Figure 1.12C) (Etemad-Moghadam *et al.* 1995; Tabuse *et al.* 1998; Hung and Kemphues 1999; reviewed in Schneider and Bowerman 2003; reviewed in Nance 2005). The restriction of the PAR-3/PAR-6/PKC 3 complex to the anterior permits PAR-1/PAR-2 expansion into a posterior domain (Severson and Bowerman 2003; Cuenca *et al.* 2003). The PAR proteins are responsible for transducing the polarity signal to downstream fate determinants to result in the formation of the anterior-posterior axis (Figure 1.12D).

Two distinct polarizing events occur in the newly fertilized embryo: cortical and cytoplasmic polarity, both of which are required for embryonic polarity (Figure 1.12B red arrows). Cortical polarity involves for the redistribution of polarity components anchored at the cortex, for example the PAR proteins. Cytoplasmic polarity is largely generated by cytoplasmic granule movement where the granules in the centre of the embryo move posteriorly to concentrate asymmetric factors such as the P-granules (Nance 2005). Contraction of the actin and myosin network causes these directional cortical and cytoplasmic flows, resulting in the redistribution and asymmetric segregation of the polarity determinants (reviewed in Golden 2000; Severson and Bowerman 2003; Cheeks *et al.* 2004; Nance 2005).

The contraction of the microfilament network is required for polarity establishment and any chemical disruption of the actin network results in a loss of anterior-posterior (A/P) polarity (Hill and Strome 1990; Hird *et al.* 1996) and cortical flows (Hird and White 1993). The actomyosin network generates contractility by the activity of myosin (NMY-2) creating the embryonic ruffling just after fertilization, recession of the ruffles towards the anterior end, formation of a deep pseudocleavage furrow during pronuclear migration, and finally the formation of distinct cytoskeletal domains. NMY-2 appears to be the sole force-generating molecule required for the contractions in the embryo that establish embryonic asymmetry which, in turn, sets up the entire embryo for its stereotyped cell divisions (Munro *et al.* 2004). Loss of NMY-2 results in polarity defects including symmetrical placement of the cleavage furrow and improper PAR protein localization (Guo and Kemphues 1996; Cuenca *et al.* 2003). The distribution of an NMY-2::GFP reporter shows that the localization pattern mirrors that described for cortical actin where NMY-2::GFP concentrates at the anterior end of the embryo and demarcates the A/P boundary and contractile region of the embryo (Figure 1.12E-G) (Strome 1986; Hird *et al.* 1996; Munro *et al.* 2004).

1.7.2 Role of maternally provided Type II myosins in the germline

The creation of germ cells is a fundamental process in the survival of any sexually reproducing species. *C. elegans* is hermaphroditic where the germline undergoes a modified form of cytokinesis to develop both sperm and oocytes (Skop *et al.* 2004) (Figure 1.13). The proliferation and development of germ cells in the hermaphrodite gonad begins with a period of mitotic proliferation of germ cell nuclei in the extreme distal region of the gonad, entry into meiosis as the nuclei proceed proximally (ie. toward the vulva) and finally the cellularization into distinct sperm or oocytes in the proximal gonad (Figure 1.13B). The germ cell nuclei mature into primordial germ cells (PGCs) when nuclei are separated by lateral membrane boundaries but remain connected to the syncytium. The nuclei are closely opposed to the gonad surface and there exists a central core, or rachus, that supplies the cytoplasm to all the partially separated primordial germ cells. The final stage to complete oocyte formation occurs in the proximal gonad and involves the complete encircling of nuclei by membranes to separate nuclei into distinct cytoplasm (reviewed in Hubbard and Greenstein, 2000).

1.7.2.1 Molecular mechanism of cellularization

It has been proposed that the cellularization machinery is similar to that used during cytokinesis, where an actomyosin based contractile apparatus invaginates the membrane between the nuclei (Skop *et al.* 2004). In agreement with this hypothesis several genes required during cytokinesis, such as myosin light chain (*mlc-4*) (Shelton *et al.* 1999) and profilin (Severson *et al.* 2002) result in hermaphrodite sterility. Some of the components of the cascade that initiates embryonic cytokinesis have been identified and many also play a role during germline cellularization. However, the major components of the contractile apparatus, namely actins and myosin heavy chains, have not been investigated for their role during germline cellularization.

One of the motor proteins necessary for cytokinesis in *C. elegans* is NMY-2, a non-muscle type II myosin (Guo and Kemphues 1996). The role of NMY-2 in the germline appears to be consistent as the molecular motor of cellularization. NMY-2 localizes to the sites of oocyte cellularization (Figure 1.13C) (Piekny and Mains 2002; Munro *et al.* 2004) and *nmy-2* RNAi treated worms produce sterile F1 progeny (Simmer *et al.* 2003; Piekny *et al.* 2003; Piano *et al.* 2002; Kamath *et al.* 2003) but the reason for this sterility was not fully investigated. It is suggested that actin and myosin are required for the formation of germ cells and are possibly the sole contractile mechanism that generates PGCs.

1.7.2.2 Cellularization in the Drosophila melanogaster embryo

Several components of the cytokinetic mechanism have been implicated in the cellularization of the *Drosophila melanogaster* blastoderm in a system potentially analogous to germline cellularization in the worm. The process of *Drosophila* cellularization involves the cellularization of syncytial nuclei during cycle 13 of embryogenesis. Cellularization proceeds through four distinct phases: 1) localization of nuclei to the interior face of the embryo and initiation of membrane barrier formation, 2) completion of nuclear elongation 3) lateral membrane growth continues through the basal end of elongated nuclei and 4) completion of cellularization (reviewed in Mazumdar and Mazumdar 2002). The actomyosin contractile apparatus forms a hexagon around each nucleus during membrane invagination forming a network over the entire embryo surface (reviewed in Mazumdar and Mazumdar 2002). Cellularization is accomplished through movement of myosin motors along the actin framework gradually reducing the diameter of the actin contractile rings and finally pinching off each nucleus into blastoderm cells. Recent studies in *Drosophila* have shown that myosin is required for cellularization, but only for the final stages, where the membrane is constricted inwards to complete the encircling of the cell instead of the formation of the lateral membranes between nuclei (Royou *et al.* 2004).

1.7.2.3 Cellularization in Caenorhabditis elegans

In *C. elegans* little research has been conducted on germline cellularization, but it can be hypothesized that mechanistically the processes between flies and worms have been conserved. Cytokinetic factors are involved in the germline and there is a growing list of genes that result in sterile hermaphrodites when mutant. These sterile hermaphrodites often have normal meiosis in the germline but fail to cellularize the nuclei and produce oocytes. For example, CYK-1 is a formin homology protein that may be required for localized modification of the membrane-associated actin cytoskeleton (Swan *et al.* 1998) not only during cytokinesis but oogenesis as well (Severson *et al.* 2002) as *cyk-1* mutants exhibit sterility.

It is not known how large a role the actin and myosin network will play in nematode cellularization. For example, it is possible that myosin may only be required for the later stages of cellularization like in flies. The presence of NMY-2 and actin along all membranes of the PGCs (Figure 1.13C) (Piekny and Mains 2002; Strome 1986) suggests that actin and myosin are required for all stages of cellularization. Cellularization likely occurs as actin and myosin (possibly NMY-2) create a cortex on which the lateral membranes can be added to grow the PGC boundaries into the centre of the gonad. Closure of the PGC windows that are formed likely involves a combination of membrane addition and constriction similar to cytokinesis (Figure 1.13C). Closure of the PGC windows only occurs at the distal to proximal turn of the gonad therefore we can hypothesize that if the machinery is the same, there are likely two distinct regulatory steps to activate lateral membrane growth and apical PGC closure.

1.8 Regulation of non-muscle myosin contraction

Myosin function must be highly regulated because contraction of myosin can induce very drastic cell shape changes and processes. The regulation of myosin is primarily accomplished by different phosphorylation events (reviewed in Bresnick 1999) and only the regulatory events of non-muscle type II myosins will be focused on here.

Inactive myosin is incapable of forming bipolar contractile filaments because the coiled-coil tail domain folds back on itself in an autoinhibitory state (Glotzer 2005) (Figure 1.14A). As discussed in section 1.1, myosin light chains self assemble onto the neck region of the myosin and these light chains act as a major regulatory site for myosin activity and their association is required for myosin assembly. The first regulator of non-muscle myosin discovered in *C. elegans* was the regulatory myosin light chain (*mlc-4)* that was shown to be the only non-muscle regulatory light chain in worms (Shelton *et al.* 1999). Loss of *mlc-4* resulted in failed cytokinesis, polarity establishment and eventual sterility (Shelton *et al.* 1999) similar to the phenotypes observed for knockdown of NMY-2 (Guo and Kemphues 1996; Cuenca *et al.* 2003). MLC-4 is the target of regulatory phosphorylation events: phosphorylation of serine 19 or threonine 18 is required for allowing myosin to interact with actin (Sakurada *et al.* 1998) (Figure 1.14B and C). Many different kinases can phosphorylate MLC including ROCK (Rho kinases), myosin light chain kinases (MLCK) and citron kinases (Matsumura 2005). In *C. elegans* ROCK (*let-502)* activity is required for proper cytokinesis and germline cellularization (Piekny and Mains 2002), however the cytokinetic defect and sterility are not as severe as a myosin knockdown (Guo and Kemphues 1996) suggesting that *let-502* is not the only kinase required for myosin activation. Several other candidates exist for potential regulators of NMY-2 in worms however one of the more promising candidates, MLCK, was recently found to be dispensible for cytokinesis in the early embryo (Batchelder *et al.* 2007). Similarly, the citron kinase gene in worms is not required for cytokinesis (Batchelder *et al.* 2007).

There are no obvious remaining candidates for molecules that regulate NMY-2 along with *let-502*. However, potential screens for redundancy with double RNAi treatments may eventually reveal these regulators of NMY-2. LET-502 acts antagonistically to a myosin phosphatase (*mel-11)* that removes the phosphorylation of LET-502 (Piekny and Mains 2002; Piekny *et al.* 2003). Depletion of this myosin regulator causes precocious cytokinesis indicating it acts as a negative regulator of non-muscle myosin activity by removing the activating phosphorylation added by LET-502 (Figure 1.15). The regulators LET-502 and MEL-11 are themselves regulated by signaling by the small GTPase Rho (Etienne-Manneville and Hall 2002). Rho-GTPases are molecular switches that can exist in either an activated GTP bound form or an inactive GDP bound form. The switch between these two activity states is catalyzed by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) (Schmidt and Hall 2002). There are many RhoGAPs present in the nematode genome (Schmutz *et al.* 2007) and a few of those have been implicated in non-muscle myosin mediated contractility. RhoGAPs turn off Rho signaling (Figure 1.15) and one RhoGAP, CYK-4, is required for hermaphrodite fertility (Kamath and Ahringer 2003). Two other RhoGAPs have been implicated as a control point for NMY-2 activity – RGA-3 and RGA-4 are redundant and nearly identical RhoGAPs required for embryogenesis but have non-overlapping functions with CYK-4 (Schmutz *et al.* 2007).

CYK-4, RGA-3 and RGA-4 represent some of the RhoGAPs present in worms and these genes act antagonistically with the GEFs that promote activation of Rho signaling. One such GEF is ECT-2 (Figure 1.15) (Motegi and Sugimoto 2006) that is required both for embryogenesis as well as germline cellularization.

The regulatory events that control non-muscle myosin activity involve multiple different inputs to activate NMY-2 in different tissues and regions within cells. LET-502 is likely not the only kinase that can phosphorylate myosin present in *C. elegans* (Piekny and Mains 2002); however no other candidates have yet been identified.

1.9 Goals of this thesis

Genetic studies suggested a maternal contribution of UNC-45 and a role for UNC-45 independent of MHC B. However, this role for UNC-45 outside the nematode body wall muscle has not previously been pursued, therefore the goal of this thesis is to identify the non-muscle processes that require UNC-45. In order to accomplish this, maternally provided UNC-45 must be depleted via both genetic and RNA interference methods. Worms depleted for UNC-45 will be examined for defects including those where a role for non-muscle Type II myosins had previously been identified including cytokinesis, embryonic polarity (Cuenca *et al.* 2003) and germline cellularization. Additionally, a characterization of the mechanistic features of cellularization has not been completed and will be addressed in the course of this thesis. Finally, the regulation of myosin in the germline has not been previously studied and provides a model to study the spatial regulation of myosin contraction during cellularization. The results obtained will contribute to our understanding of UNC-45 as a myosin chaperone and will expand the role of UNC-45 to include non-muscle roles.

1.9.1 Implications of UNC-45 research

The fact that UNC-45b is upregulated in some forms of human cancer (Bazzaro *et al.* 2007) suggests that UNC-45 has some fundamental roles in maintaining myosin homeostasis. The importance of human UNC-45 may also include specific roles for the UNC-45a homolog in conditions such as cardiomyopathies and muscle related diseases.

There are many human muscle diseases that result from mutations in sarcomeric proteins called myopathies that include mutations in both of the major components of the sarcomere – actins and myosins (reviewed in Bonneman and Laing 2004). Some of these myopathies have been suggested to be caused by a loss of muscle organization or accumulation of aggregates. This includes a hyaline inclusion body myopathy that is characterized by aggregates of myosin likely from a failure to assemble the thick filaments. Patients with these myopathy characteristics contain a missense mutation in a type II skeletal myosin (*MYH7*) in the tail domain of myosin that facilitates thick filament formation (Tajsharghi *et al.* 2003; Bohlega *et al.* 2004; Darin *et al.* 2007). Diseases such as myotonic dystrophy and other myopathies have been shown to require chaperones possibly to facilitate assembly or to prevent aggregation of sarcomeric components (Brown, D.D. *et al.* 2007). UNC-45 in human cell culture lines has been shown to interact with CDC-48 and CDC-48 has been implicated in inclusion-body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) (Janiesch *et al.* 2007) suggesting that UNC-45 may also play a significant role in muscle related illnesses. Additionally, as humans age there is a marked decrease in muscle strength and efficiency called sarcopenia. This gradual muscle weakness may be a result of decreased chaperone activity (Doran *et al.* 2007).

The function of UNC-45 in the muscle cells of *C. elegans* contributes to our understanding of the role of this chaperone. However, crucial functions for UNC-45 lie outside the body wall muscle; for instance, in the germline and embryo. The nematode provides a unique advantage to other model organisms to study embryonic processes because the embryos develop *ex utero* and are transparent. Studies done by our lab and others have contributed to our knowledge about the function of UNC-45 in the muscle which can also be used to study chaperone activity in other developmental processes. Similarly, roles for UNC-45 outside the muscle have been found in vertebrate systems. This includes a function for a general cell isoforms of UNC-45 in ovarian cancer progression and metastasis in human cell culture lines (Bazzaro *et al.* 2007). In this thesis I will demonstrate that UNC-45 is required outside the muscle.

1.10 Figures

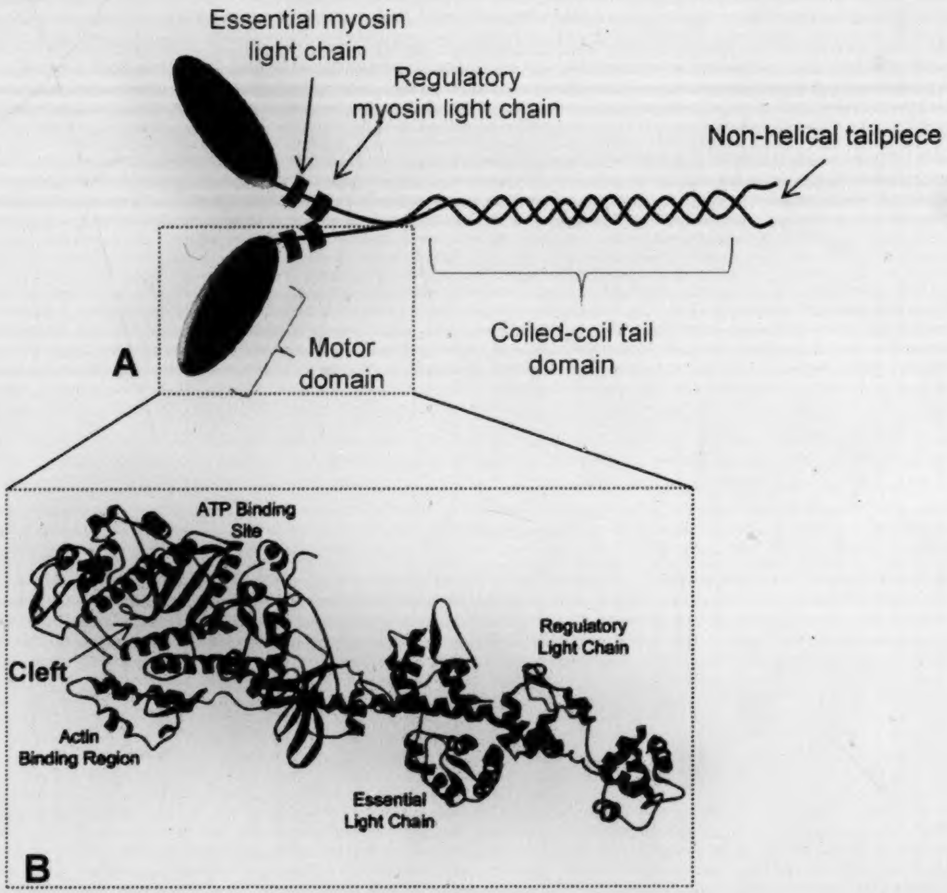

Essential myosin light chain
Regulatory myosin light chain
Non-helical tailpiece
Coiled-coil tail domain
Motor domain
A
ATP Binding Site
Cleft
Actin Binding Region
Essential Light Chain
Regulatory Light Chain
B

Figure 1.1. Structure of type II myosins. A) Schematic of type II muscle and non-muscle myosin dimers. Type II myosins contain a myosin heavy chain dimer (blue) and two light chains (red and green) that associate at the neck region. The coiled – coil tail region facilitates dimerization and the non-helical tailpiece facilitates assembly of dimers into antiparallel structures. B) Illustration of the structure of the myosin head domain (adapted from dir.nhlbi.nih.gov/labs/lmc/cmm/myosinlab.asp).

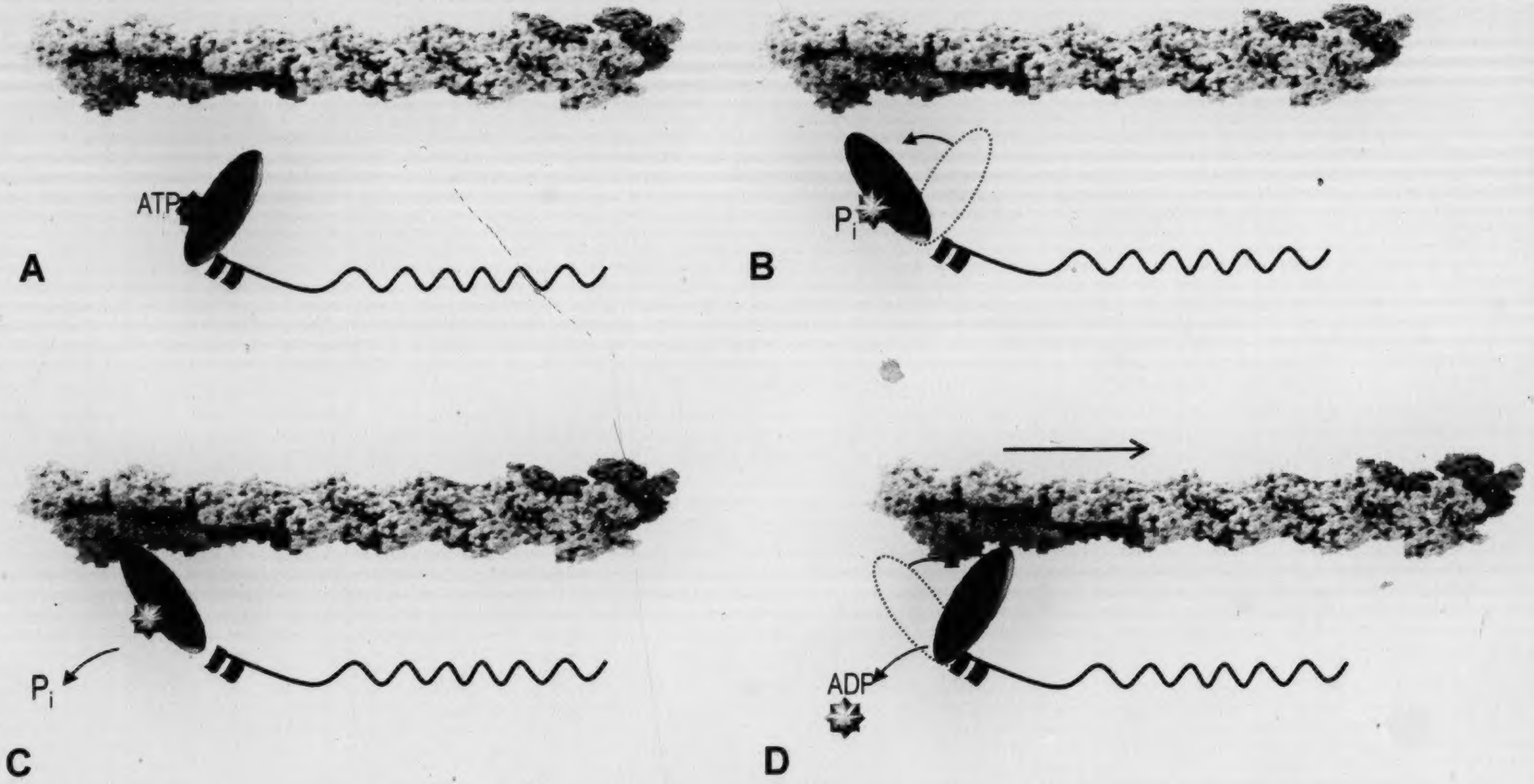


Figure 1.2. Contractile cycle of myosin. A) ATP binding stimulates release of myosin head off the thick filament. B) ATP hydrolysis results in a conformational change. C) Release of the inorganic phosphate promotes actin binding. D) Release of ADP results in myosin contraction pulling the thin filament with it. Diagram of thin filament adapted from The National Library of Medicine (www.nlm.nih.gov/).

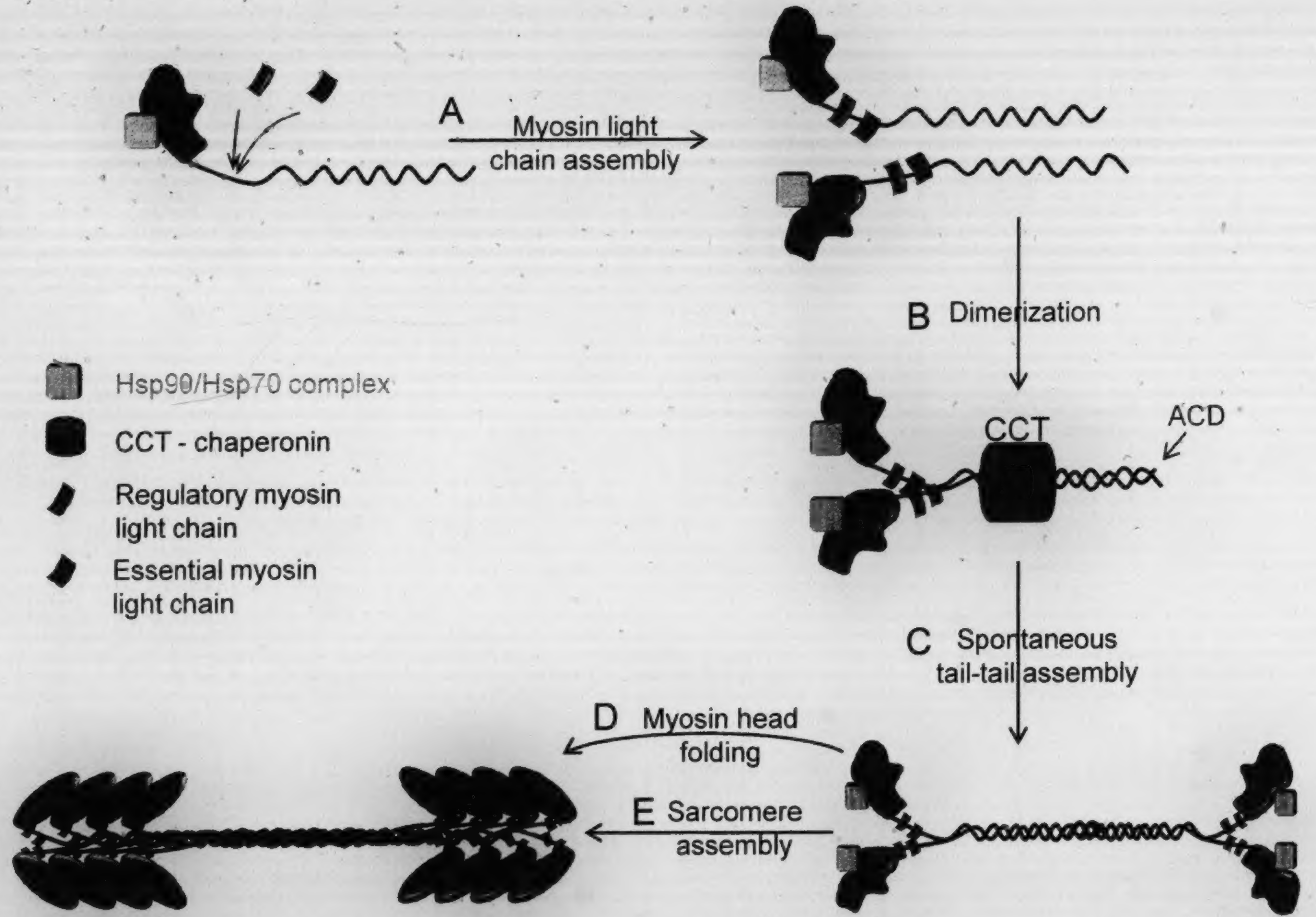

A
Myosin light chain assembly
B Dimerization
CCT
ACD
C Spontaneous tail-tail assembly
D Myosin head folding
E Sarcomere assembly
Hsp90/Hsp70 complex
CCT - chaperonin
Regulatory myosin light chain
Essential myosin light chain

Figure 1.3. Myosin maturation. A) Nascent myosin molecules associate with the Hsp70/Hsp90 complex (yellow). Myosin light chain assembly (green and red) and folding of the tail region occurs rapidly. B) Dimerization of the myosin tails may require the chaperonin CCT. C) The assembly competence domain at the extreme C-terminus (ACD) promotes tail-tail dimer formation. D) Myosin head folding is the rate limiting step and can occur at any point in this cascade. E) Assembly into sarcomeres likely requires many different factors but does not require properly folded myosin heads.

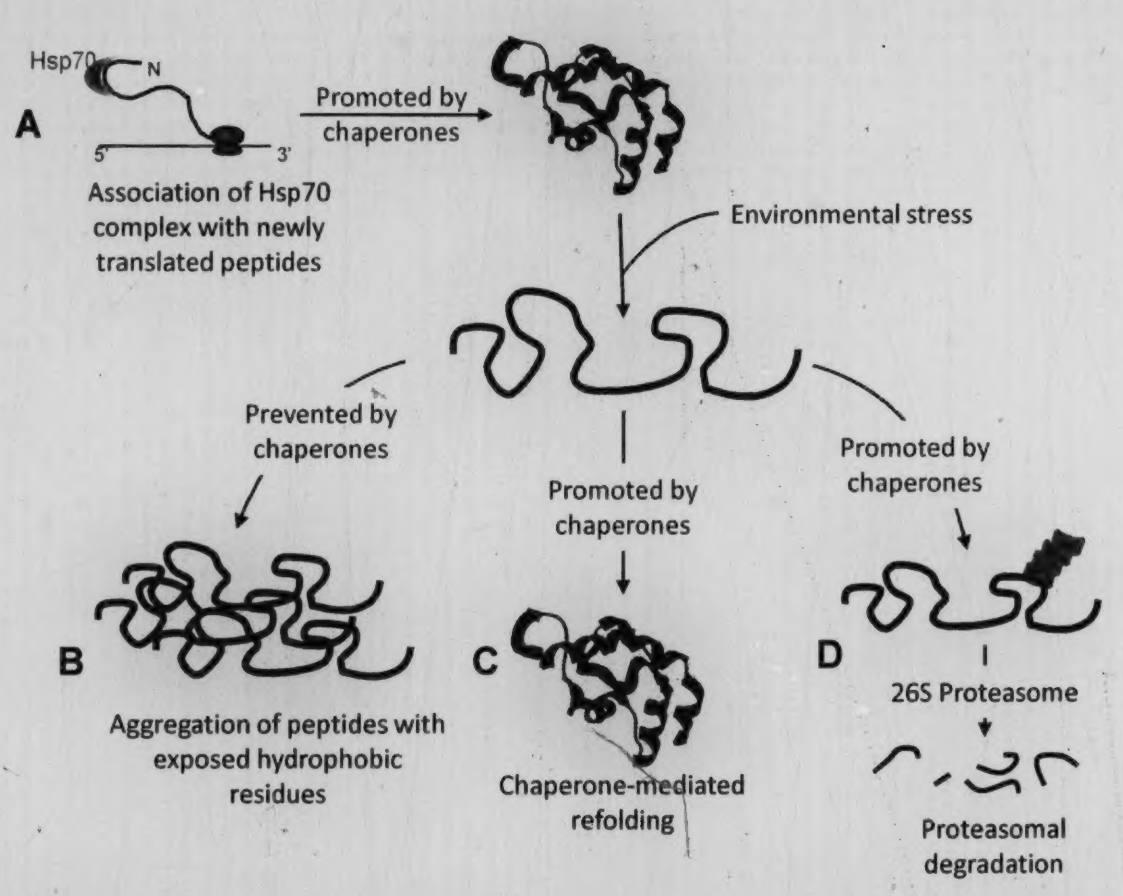

Hsp70
N
A
5'
3'
Promoted by
chaperones
Association of Hsp70
complex with newly
translated peptides
Environmental stress
Prevented by
chaperones
Promoted by
chaperones
Promoted by
chaperones
B
C
D
26S Proteasome
Aggregation of peptides with
exposed hydrophobic
residues
Chaperone-mediated
refolding
Proteasomal
degradation

Figure 1.4. Chaperone function. Molecular chaperones ensure the protein quality in the cell by either: A) promoting proper folding of nascent polypeptides, B) Preventing aggregation upon induced misfolding, C) Promoting refolding after induced misfolding or D) targeting terminally misfolded proteins for degradation by the proteasome.

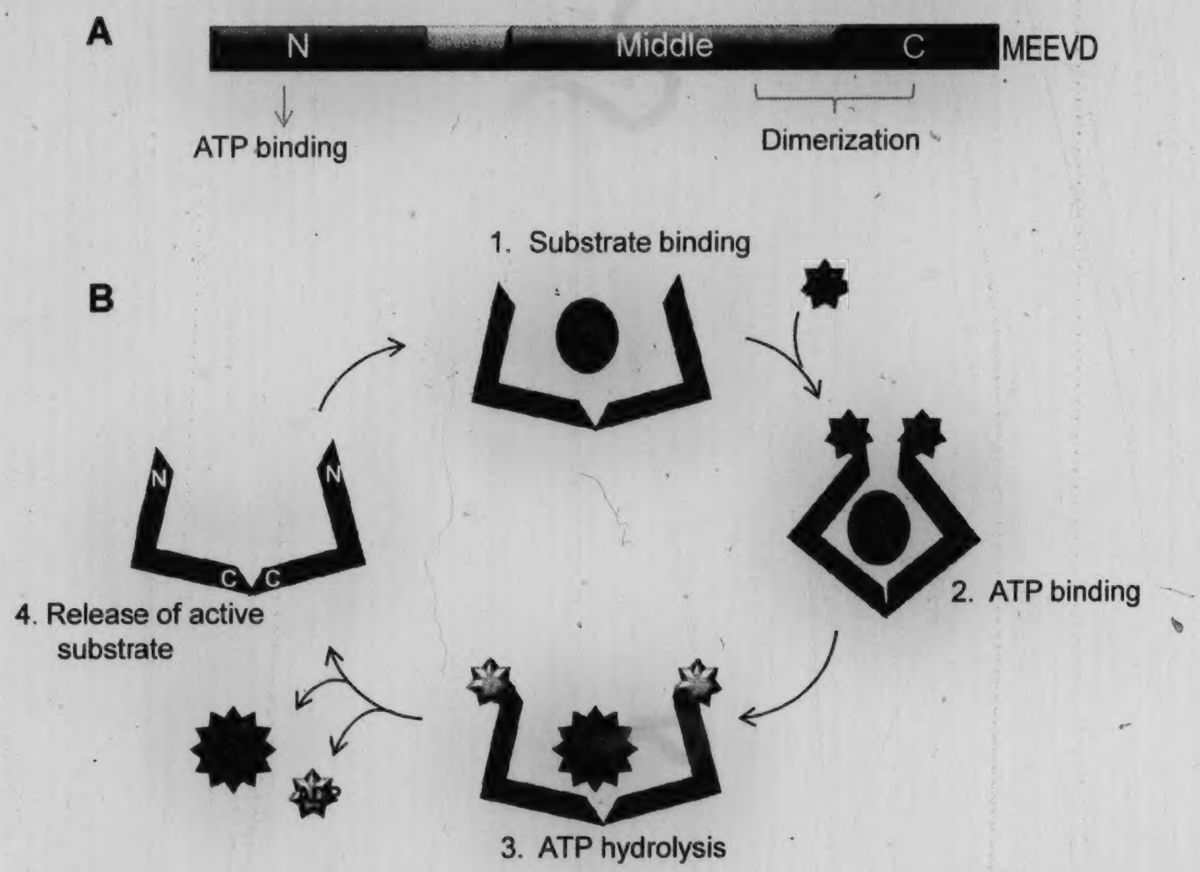

A
N
Middle
C
MEEVD
ATP binding
Dimerization
B
1. Substrate binding
2. ATP binding
3. ATP hydrolysis
4. Release of active substrate
N
N
C
C

Figure 1.5. Hsp90 structure and function. A) Schematic of Hsp90 protein domains (adapted from Young *et al.* 2001). B) Chaperone cycle of Hsp90.

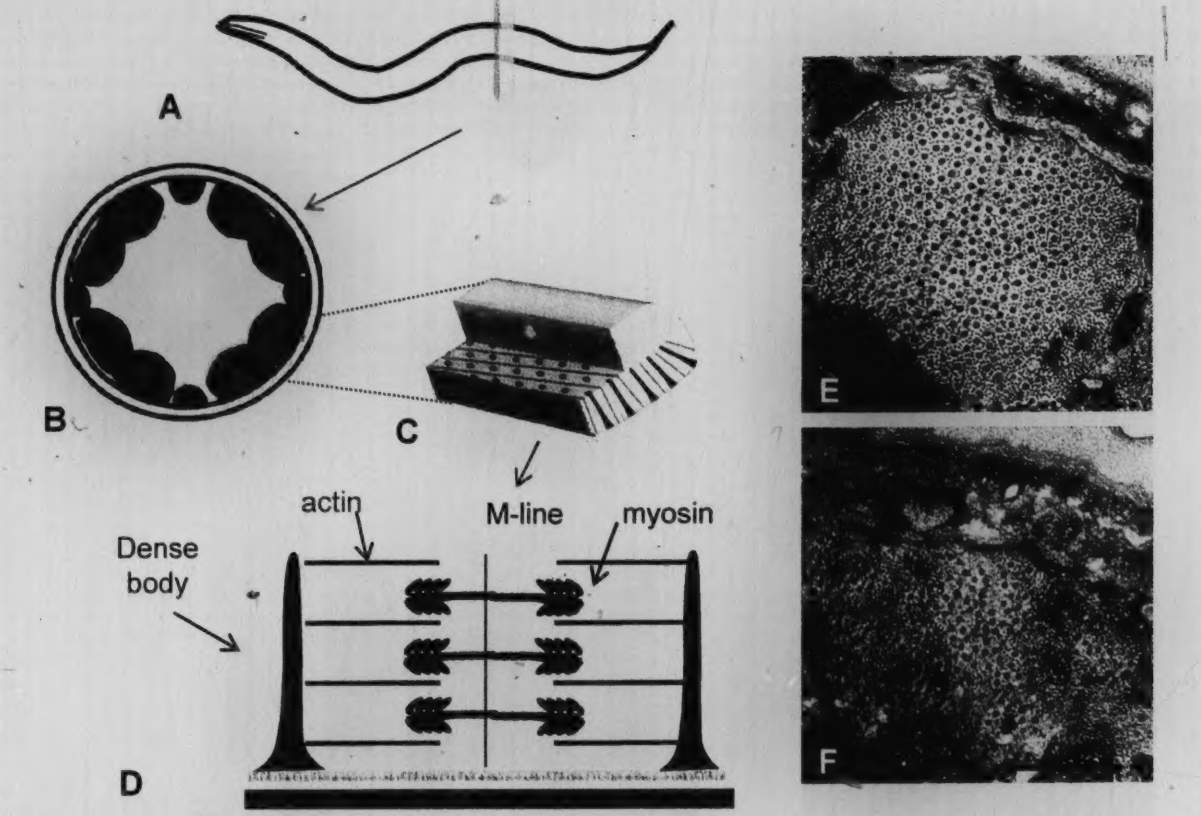


Figure 1.6. Muscle structure in *C. elegans.* A) Cross section of a nematode body cavity. B) Body wall muscles are composed of four quadrants lining the body cavity of the worm. C) Orientation of the sarcomeres in the body wall muscles (adapted from Moerman *et al.* 1997). D) Structure of the sarcomere. Thick filaments are interdigitated with actin containing thin filaments. Thin filaments are anchored in the dense bodies and during contraction the length of the sarcomere decreases. E) Cross section of wildtype myosin thick filament organization shown by dark grey circles (adapted from Barral *et al.* 1998). F) *unc-45* mutant animals show decreased number and organization of thick filaments (adapted from Barral *et al.* 1998).

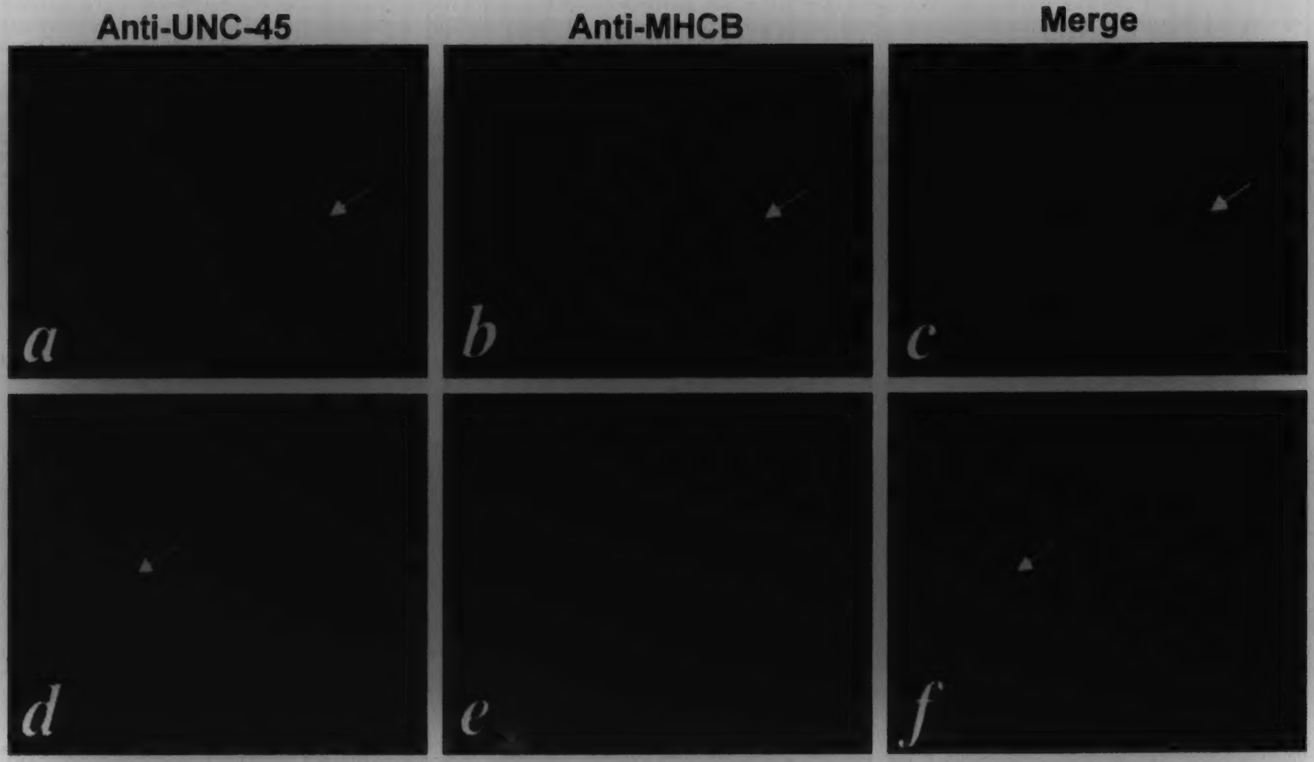


Figure 1.7. Colocalization of UNC-45 with MHCB in the adult nematode body wall muscle. A) Localization of UNC-45 to the thick filaments (arrow). B) MHC B staining to the thick filaments (arrow). C) Colocalization of MHC B with UNC-45 (arrow). D) UNC-45 localization pattern to the outside of the thick filaments (arrow). E) MHC A localizes to the central region of the thick filaments. F) No co-localization of UNC-45 and MHC A (arrow). Adapted from Ao and Pilgrim 2000.

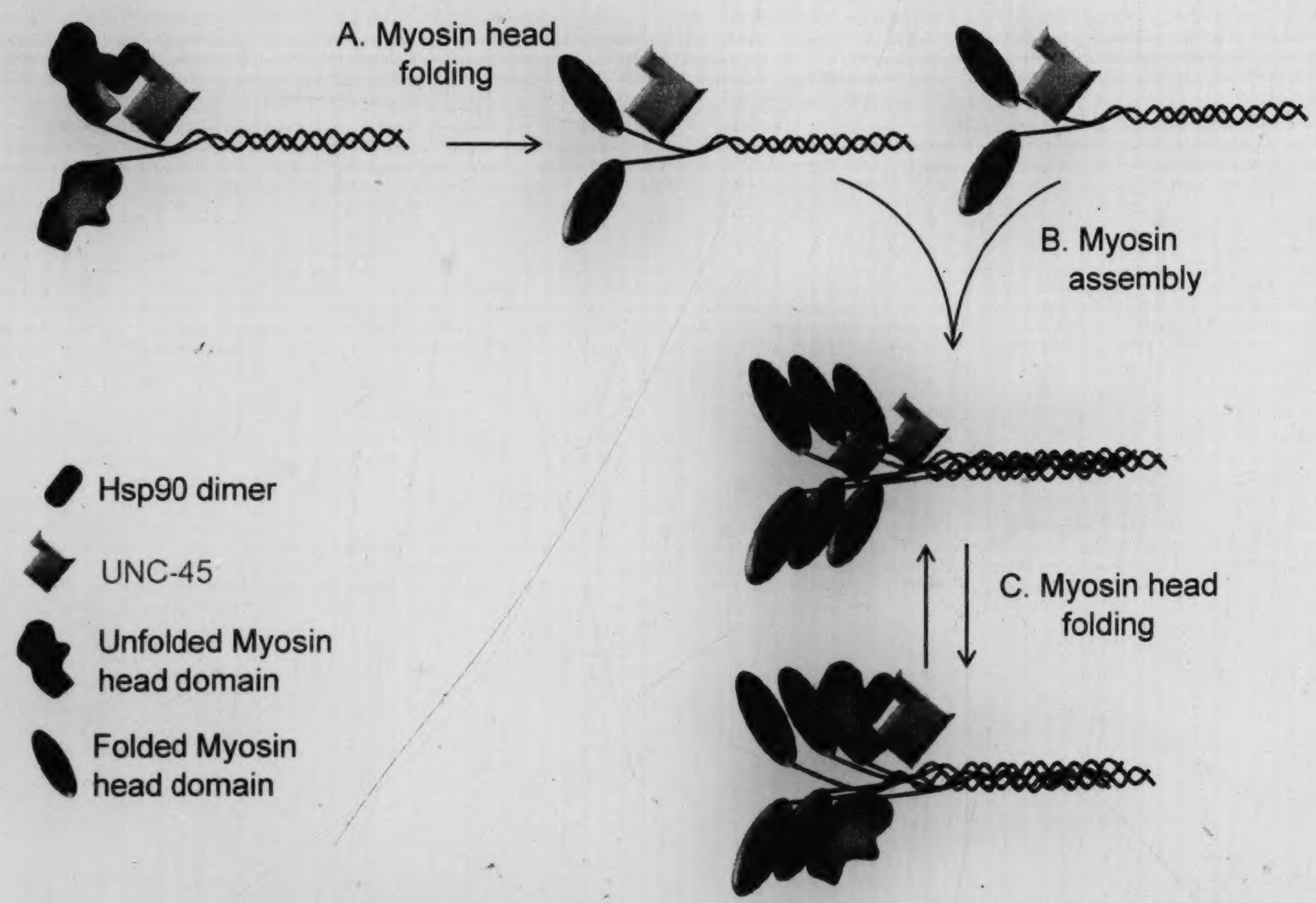

A. Myosin head folding
B. Myosin assembly
C. Myosin head folding
Hsp90 dimer
UNC-45
Unfolded Myosin head domain
Folded Myosin head domain

Figure 1.8. Possible roles for UNC-45. A) UNC-45 and the Hsp90 co-chaperone may be responsible for folding the globular myosin head domain. B) UNC-45 may promote myosin assembly into thick filaments. C) UNC-45 and Hsp90 may refold myosin heads that have unfolded during contraction.

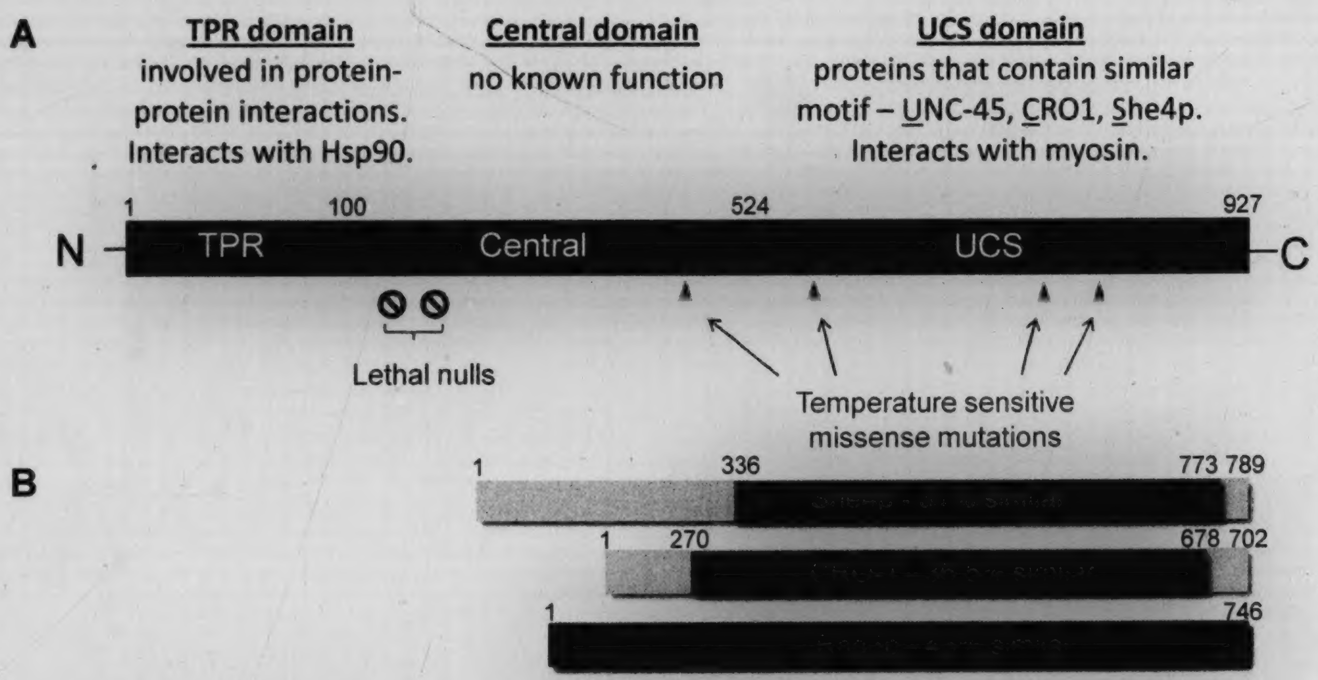

A
TPR domain
involved in protein-protein interactions. Interacts with Hsp90.
Central domain
no known function
UCS domain
proteins that contain similar motif – UNC-45, CRO1, She4p. Interacts with myosin.
1
100
524
927
N
TPR
Central
UCS
C
Lethal nulls
Temperature sensitive missense mutations
B
1
336
773 789
1
270
678 702
1
746

Figure 1.9. Structure of the UNC-45 protein and its fungal homologs. A) *C. elegans* UNC-45. The N-terminal TPR domain (purple) is involved in protein-protein interactions. The central domain (green) has no known function. The C-terminal UCS domain contains the region that interacts with myosin and is common to other UNC-45 homologs. Location of lethal (nonsense) alleles are noted by the (⊘) symbol and the location of the known temperature-sensitive alleles are noted by the (▾) symbol. B) UCS containing fungal homologs. She4p from *Saccharomyces cerevisiae,* Cro-1 from *Podospora anserina* and Rng3p from *Schizosaccharomyces pombe*. Dark blue is the region compared to UNC-45 for similarities.

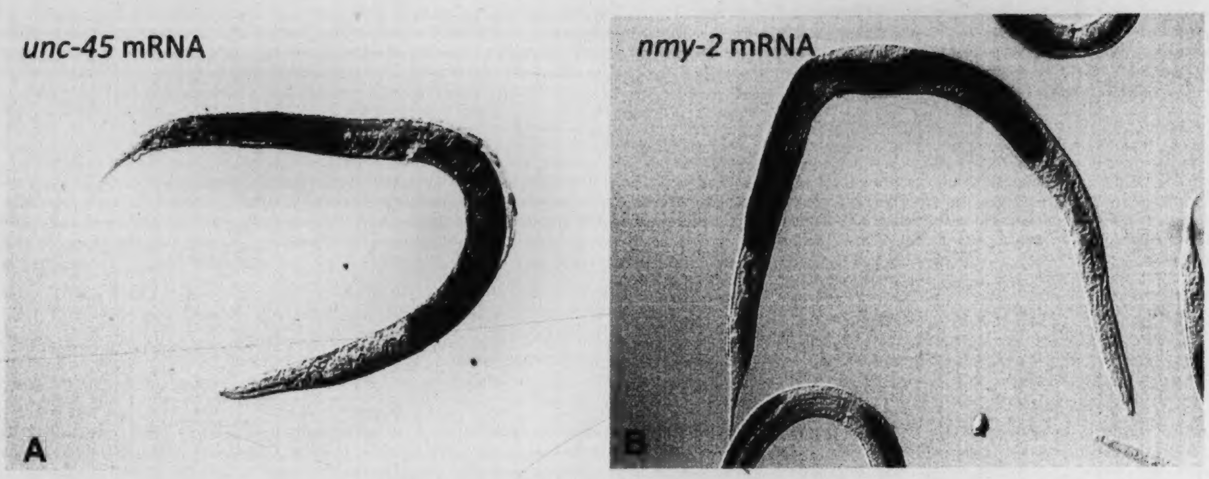


Figure 1.10. Maternal expression of *unc-45* and *nmy-2*. A) *unc-45* mRNA is expressed throughout the adult hermaphrodite germline. B) *nmy-2* mRNA is expressed throughout the adult hermaphrodite germline. (Images adapted from NextDB http://nematode.lab.nig.ac.jp/db2/KeysrchForm.php. Search NMY-2 or UNC-45)

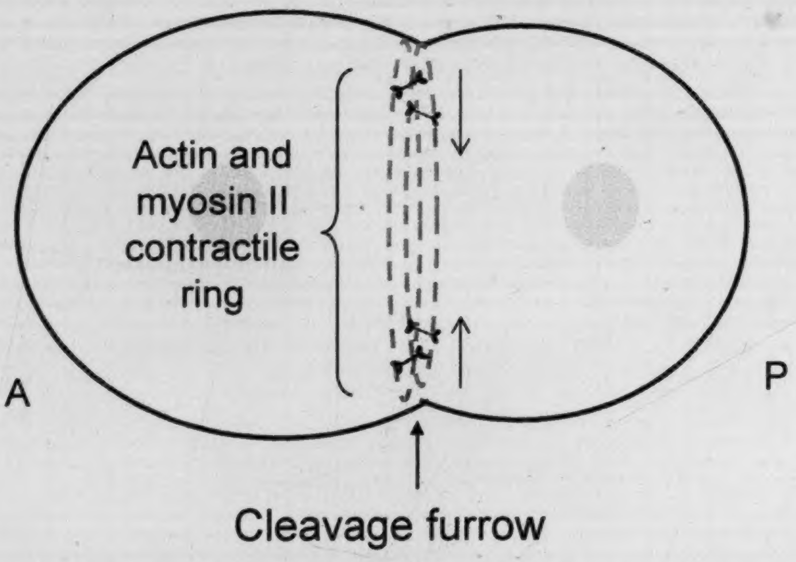

Actin and myosin II contractile ring
A
P
Cleavage furrow

Figure 1.11. Actomyosin ring during cytokinesis. During cytokinesis the actin filaments (purple dashed line) and non-muscle myosin II (green) accumulate at the cortex into a contractile ring. Upon activation of myosin the diameter of the ring decreases (blue arrows) thus separating cytoplasm of daughter cells into two distinct compartments. A and P denote anterior and posterior poles.

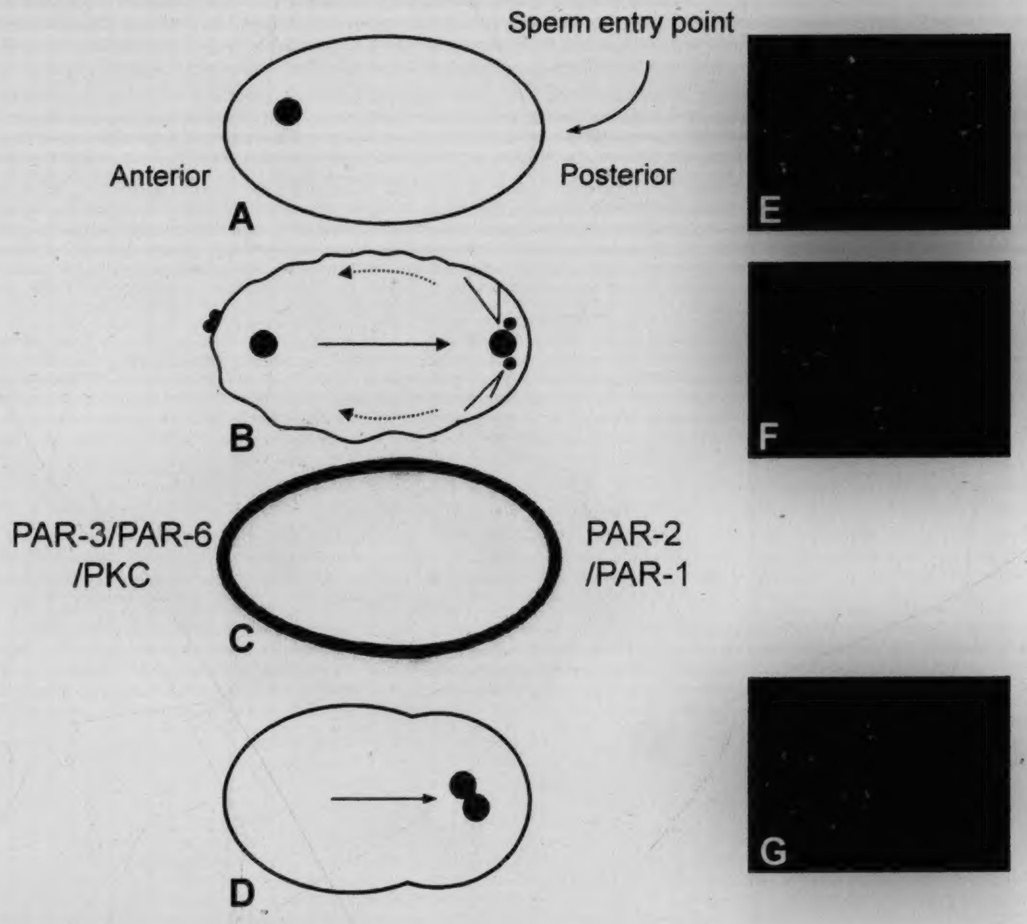


Figure 1.12. Polarity establishment and myosin dynamics in the one cell embryo. A) Sperm entry point determines the posterior of the embryo. B) Myosin contractile activity promotes cortical and cytoplasmic flows that redistribute polarity components. C) Cortical polarity concentrates PAR-3/PAR-6/PKC complex in the anterior and PAR-1 and PAR-2 in the posterior. D) Asymmetry of polarity determinants result in asymmetric pronuclear meeting. E) NMY-2::GFP image shows myosin foci distributed throughout cortex. F) Asymmetric contraction results in anterior accumulation of NMY-2::GFP. G) Cortical asymmetry is maintained. (Images E-G adapted from Munro 2004).

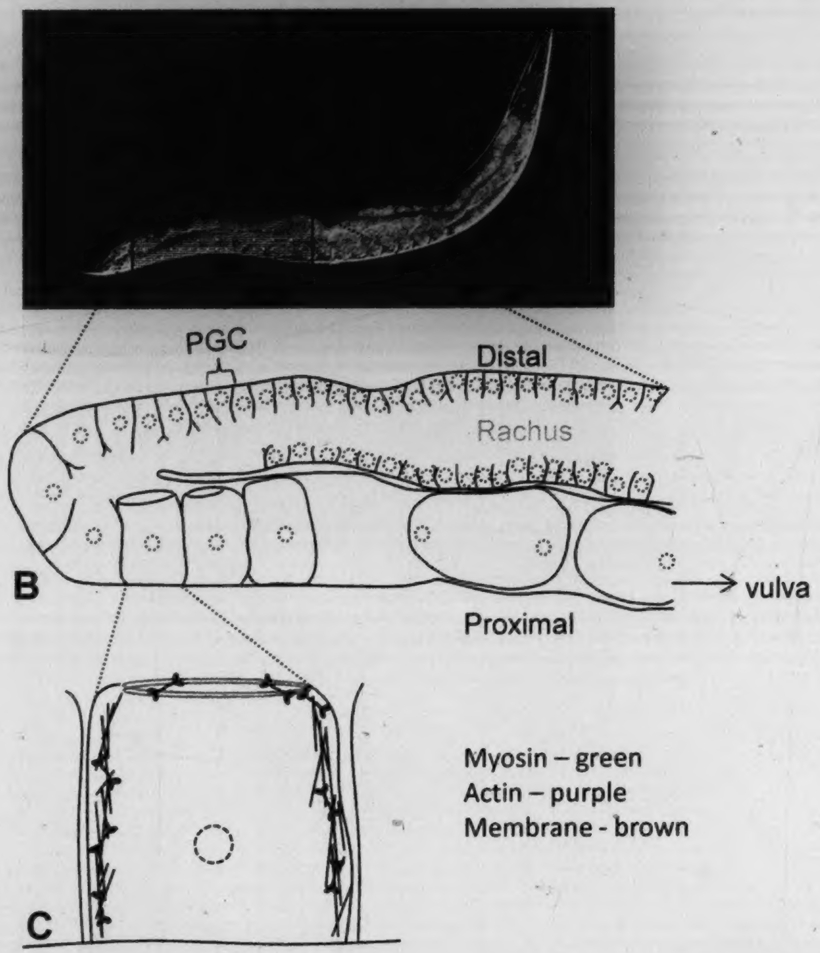

PGC
Distal
Rachus
B
Proximal
vulva
C
Myosin – green
Actin – purple
Membrane - brown

Figure 1.13. Structure of the hermaphrodite gonad. A) Adult hermaphrodite. B) Schematic of the germline. Germ cell nuclei are syncytial through the distal germline and become cellularized at the distal to proximal turn of the gonad. C) Actin and myosin are distributed along the cellularization membranes and surround the window of the syncytium.

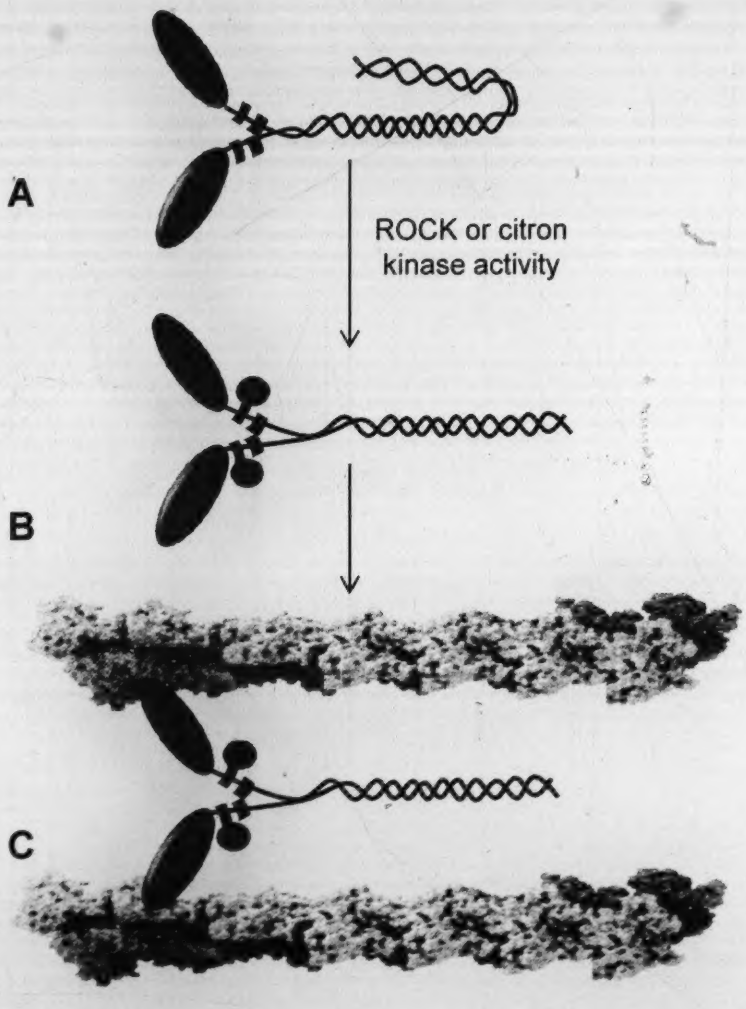


Figure 1.14. Myosin regulation. A) Fully assembled myosin in the autoinhibited state. B) Phosphorylation of the regulatory myosin light chains activates myosin. C) Activated myosin can bind actin during a normal ATP-dependent contractile cycle. Microfilament image taken from The National Library of Medicine (www.nlm.nih.gov/).

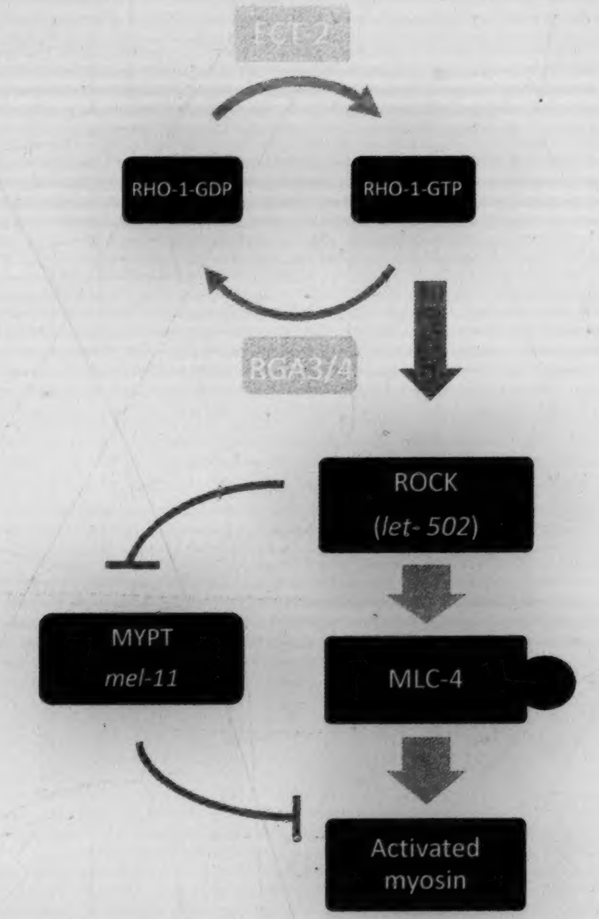

ECT-2
RHO-1-GDP
RHO-1-GTP
RGA3/4
ROCK
(*let- 502*)
MYPT
mel-11
MLC-4
Activated
myosin

Figure 1.15. Myosin regulatory pathway. Myosin is regulated by phosphorylation of the regulatory light chain (MLC-4) which can be phosphorylated by a Rho cascade and Rho-associated myosin light chain kinase (ROCK/*let-502*).

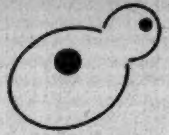
Organism	UCS protein	Myosin interaction	Defining phenotype	Predicted role for UCS protein
Saccharyomyces cerevisiae 	She4p	Myo5p (Type I) Myo4p (Type V)	ASH1 mRNA mislocalization. Disorganized actin cytoskeleton	Mediate myosin localization
Podospora anserina 	CRO1	None identified to date	Failure to switch from syncytial to cellular fate	Recruit myosin to actin cytoskeleton
Schizosaccharomyces pombe 	Rng3p	Myo2p (Type II)	Cytokinesis defect	Assembly and localization of myosin to contractile ring

Table 1.1. Summary of fungal UCS proteins. Three fungal homologs share similarity with UNC-45 – She4p from budding yeast, CRO1 from *Podospora anserina* and Rng3p from fission yeast. All three homologs play a role in myosin based processes.

1.11 References

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2. Maternal UNC-45 is involved in cytokinesis and co-localizes with a non-muscle myosin in the early *Caenorhabditis elegans* embryo.[1]

2.1 Introduction

Chaperones shown to promote proper myosin folding include UNC-45 (Barral *et al.* 2002), and a similar function is proposed for proteins from other systems that share sequence similarity in the C-terminal domain of UNC-45 (Hutagalung *et al.* 2002), recently termed the UCS (UNC-45/CRO-1/She4p) family of proteins. There is evidence from in vitro studies that UNC-45 can interact with myosin in pull-down assays (Barral *et al.* 2002) suggesting that the co-localization of MHC B with UNC-45 in vivo may be due to a direct interaction. These observations have led to the conclusion that UNC-45 acts directly to ensure proper folding of MHC B in the body wall muscles.

In addition to the effect on MHC B in the larval and adult body wall muscle, UNC-45 must have another role during embryonic development. Null alleles of *unc-54* (MHC B) are paralyzed but viable and fertile (Epstein and Thomson 1974). In contrast, some nonsense alleles of *unc-45* result in embryonic lethality (Venolia and Waterston 1990; Barral *et al.* 1998). Thus, the phenotype of embryos carrying strong *unc-45* alleles is much more severe than the phenotype of embryos lacking MHC B, resulting in a paralyzed at twofold phenotype (Pat). *unc-54* null mutations do not suppress the *unc-45* embryonic lethality, as would be expected if the only role of UNC-45 was to control the assembly or stability of MHC B (Venolia and Waterston 1990).

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[1] *A version of this chapter has been published.*

Kachur, T. Ao, W., Berger, J. and Pilgrim, D. 2004. Journal of Cell Science. 117(22):5313-22.

Wanyuan Ao provided much of the preliminary data including all of figure 2.1, the yeast two hybrid screen and figure 2.4.

Jeffery Berger confirmed the data in figure 2.4 but his images were not in the final published version.

I provided the data on the cytokinetic defect (Figure 2.3) as well as the quantitative beta-galactosidase assay data (Figure 2.2) and completed all of the writing.

The Pat phenotype of *unc-45* null mutants may not be related to muscle at all. Muscle cell components are normally produced zygotically, but embryonic-lethal alleles of *unc-45* show terminal phenotypes that are dependent on the genotype of the mother, suggesting that *unc-45* product is contributed through the oocyte (Venolia and Waterston 1990). *C. elegans* non-muscle type II myosins are known to play an essential role in embryonic cytokinesis (Guo and Kemphues 1996; Shelton *et al.* 1999), and it is tempting to speculate that UNC-45 may interact with non-muscle myosin molecules in this process. This could be tested by examining cytokinesis in embryos depleted for UNC-45. Therefore, we examined the interaction of UNC-45 with non-muscle myosin II (NMY-2) and characterized the embryonic requirement for UNC-45 during cytokinesis.

2.2 Materials and Methods

2.2.1 Strains and genetics

C. elegans strain N2 Bristol (wild type) was obtained from the stock collection of the MRC Laboratory of Molecular Biology, Cambridge, UK and maintained as described (Wood 1988). The alleles of *unc-45* have been described previously (Table 2.1)(Venolia and Waterston 1990; Barral *et al.* 1998; Ao and Pilgrim 2000). The *hum-2* deletion strain RB801 *hum-2(ok596)* was generated as part of the *C. elegans* Gene Knockout Consortium, and was a gift of Robert Barstead (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). The yeast strain PJ69-4A along with the expression vectors for the two-hybrid screen were kindly provided by Dr. Philip James (University of Wisconsin, Madison, WI, USA) and maintained as described (James *et al.* 1996). All yeast transformations were done using the high efficiency lithium acetate method (Gietz and Schiestl 1995).

2.2.2 Transgenic animals

The construct (pDP#WA036), which contains the full-length *unc-45* cDNA fused in-frame to green fluorescent protein (GFP) behind the *unc-45* promoter, was described previously (Venolia *et al.* 1999). The transgenic line (DP193 *edEx74*) generated as described (Mello *et al.* 1991) contains this construct as part of an extrachromosomal array along with the plasmid pRF4 (*rol-6* [*su1006dm*]). An isolate of this line showing transmission of the array to more than 95% of the progeny was used in this study. GFP expression was examined using the FITC (fluorescein isothiocyanate) filter set on an Axioskop (Carl Zeiss) microscope.

In a second line, homozygous *unc-45(r450ts)* animals were injected with a mixture of plasmids pDP#WA036 and a *myo-3p*::GFP marker supplied by Dr. Andy Fire (pPD118.20) (Carnegie Institute of Washington, Baltimore, MD, USA) and scored for *unc-45* rescue at the restrictive temperature. Rescued animals were then crossed with strain DP246 carrying a balanced lethal *(st601)* allele of *unc-45*. F1 heteroallelic early adults *(r450ts/st601let)* were grown at the restrictive temperature overnight, dissected and placed onto 3% agarose pads; embryos remained in the uterus of dissected adults to protect the fragility of early embryos.

2.2.3 Two-hybrid screen

Full length *unc-45* cDNA was amplified from pDP#WA036 by PCR using the primers 5' TTTCCCGGGATGGTTGCTCGAGTACAGACT 3' and 5'CAACCCGGGTTCCTGAATGGTGCTCATTTG 3'. The PCR product was digested with *Sma* I and cloned into pGBDU-C1 (James *et al.* 1996), to give plasmid pDP#WA039. This was used as a bait to screen about 10^6 transformants of a *C. elegans* cDNA mixed stage expression library in the λACT-RB1 vector, (kindly provided by Dr. Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA). Three strong positive clones and several weaker ones were identified. These were re-tested by purifying the plasmid DNAs and re-transforming them into yeast. As controls, the bait plasmid pSE1112 (Durfee *et al.* 1993), which encodes the SNF1 fusion protein, and

FEM-2 (Pilgrim *et al.* 1995) were tested. After sequencing the cDNA fragments and re-cloning into pGAD vectors, their interactions with UNC-45 were confirmed a third time. The two positive cDNAs that will be discussed are a 1.6 kbp fragment of *nmy-2* and a 420 bp fragment of *hum-2*. A third strong positive, *Ce*UFD2 required the TPR domain for an interaction and was removed from consideration.

To test different domains of UNC-45 with these positives, *unc-45* subclones were constructed. A 2.6 kbp *unc*-45 cDNA fragment was amplified by PCR from pDP#WA039 using primers 5' CAACCCGGGTTCCTGAATGGTGCTCATTTG 3' at the 3' end of *unc-45* and 5' GATCCCGGGATTGTTGAAGTTCTTCAG 3' at the 5' end of exon 4. It was cloned into pGBDU-C1, resulting in plasmid pDP#WA040 which encodes a truncated UNC-45 protein lacking the TPR domain (the first 112 amino acids at the N-terminus).

Quantification of the yeast two-hybrid interactions was performed using the Pierce Quantitative β-Galactosidase Assay kit according to kit specifications.

2.2.4 RNA interference (RNAi)

RNA mediated interference has been shown to be a useful tool for phenocopying a null mutation in many genes (Fire *et al.* 1998; Montgomery *et al.* 1998; Tabara *et al.* 1998). The partial *nmy-2* cDNA was re-cloned into pBluescript KS$^-$ (Stratagene). Double-stranded RNA was prepared using a MEGAscript T7 in vitro transcription kit (Ambion) using the primers 5' AGCTCGGTAATACGACTCACTATAGGGAAC 3' and 5' CCAGTGAATTGTAATACGACTCACTAT 3'. After microinjection of the RNA into the gonad of adult hermaphrodites, the same phenotype for *nmy-2* was observed in the progeny as previously described (Guo and Kemphues 1996). To generate RNAi-treated worms for immunostaining, a soaking method was used (Tabara *et al.* 1998; Timmons and Fire 1998). Briefly, 1 µl of Lipofectin (Gibco-BRL) and 4 µl of dsRNA prepared as above were mixed and incubated at room temperature for 15 minutes. 15 to 20 L4-stage hermaphrodites were added to the dsRNA-liposome mixture and incubated for 10- 24 hours at room temperature. Following incubation, the worms were transferred to an agar plate with *E. coli* (strain OP50) and resulting progeny were examined as described below.

2.2.5 Immunofluorescence microscopy

Embryos from either wildtype or *nmy-2* RNAi-treated hermaphrodites were fixed as described (Miller and Shakes 1995) using methanol/acetone followed by air-drying. To stain the adult gonads, the worms were placed on a slide in M9 buffer and cut beneath the pharynx to release the intact gonads from the worm bodies. These were stained using the same protocol. Mouse monoclonal antibody 5-8 against MHC B (kindly provided by Dr. David Miller III, Vanderbilt University, Nashville, TN, USA) was used at 1:1000 dilution and mouse monoclonal MH27 (provided by Dr. Joel Rothman, University of California Santa Barbara, CA, USA; Francis and Waterston 1985), was used at 1:1000 dilution. Rabbit polyclonal antisera against NMY-2 (a gift from Dr. Ken Kemphues, Cornell University, Ithaca, NY, USA) was used at 1:200 dilution and rabbit polyclonal antisera 7N5 against UNC-45 (Ao and Pilgrim 2000) was used at 1:500-1,000 dilution. Preimmune serum from the same rabbit (7N5) was used as an UNC-45 negative control. The secondary antisera (Sigma) were FITC-labeled anti-rabbit or TRITC -labeled anti-mouse (both at 1:1,000 dilution). For the UNC-45 + NMY-2 co-localization experiment, UNC-45 antibody 7N5 was directly labeled with FITC as described (Harlow and Lane 1999). The FITC-labeled antisera was used at a 1:50 dilution. DAPI was used at 1 μl/ml in the mounting media. The immunofluorescence images were taken using an Axioskop (Carl Zeiss) or by confocal microscopy (Molecular Dynamics 2001) and processed using Photoshop 5.0 (Adobe).

2.3 Results

2.3.1 UNC-45 protein is contributed to the embryo through the maternal germline

The maternal effect that is seen with strong alleles of *unc-45* predicts that *unc-45* mRNA or protein (or both) are contributed though the maternal germline. In situ hybridization shows that *unc-45* mRNA is enriched in the gonad of adult worms as well as being quite strong in 2- and 4-cell embryos (Dr. Y. Kohara, online database at http://nematode.lab.nig.ac.jp/dbest/srchbyclone.html, EST yk44f2). In older embryos, the mRNA is localized in the presumptive muscle quadrants and the protein is also detectable in these tissues. Figure 2.1 shows UNC-45 staining in the hermaphrodite gonad distal arm (Figure 2.1A) and in all cells of the early embryo up until the stage at which morphogenesis begins (Figure 2.1C-E). While staining is visible throughout the cell, it is especially apparent at the earlier stages and appears to be concentrated at the cell cortex (Figure 2.1C, D). Since transcription of zygotic genes does not usually begin until several cell cycles have passed (Seydoux *et al.* 1996; Seydoux and Dunn 1997), this is consistent with maternal contribution of UNC-45 protein and mRNA to the oocyte, and persistence at least until gastrulation. We can indirectly determine the time of onset of zygotic *unc-45* transcription by examining reporter genes. Transgenic arrays are often silenced in the *C. elegans* germline (Kelly *et al.* 1997); thus, reporter gene expression from such an array will usually only represent the zygotic somatic component. We have seen GFP expression only in the muscle cells and not in either the germline or oocytes from *unc-45::GFP* reporter constructs (Venolia *et al.*

1999; Figure 2.1I) and expression is detected prior to appearance of MHC B at the bean stage of embryogenesis (data not shown). Consistent with this, UNC-45 protein is detected by immunostaining prior to detection of MHC B (Figure 2.1K, L). At 270 minutes after fertilization, maternal UNC-45 is evenly distributed in all cells (Figure 2.1E) but after 300 minutes, UNC-45 staining is more concentrated in muscle cells (the lateral bands of muscle cells are apparent in Figure 2.1G). At the 1.5-fold stage of elongation, the zygotic expression of UNC-45 is clearly co-localized with MHC B in muscle cells (Figure 2.1M, N). Other muscle structural proteins are first detectable in the same regions at about this time (Epstein *et al.* 1993; Hresko *et al.* 1994; Moerman and Fire 1997). While it is not possible to exclude the possibility that maternal UNC-45 becomes concentrated in the muscle cells at later stages, a simpler explanation is that maternal UNC-45 protein persists in all cells of the embryo up until gastrulation. As the embryo begins to elongate, maternal protein staining disappears and zygotic UNC-45 is synthesized in a muscle-cell specific manner.

2.3.2 UNC-45 is required for proper cytokinesis during embryogenesis

Since zygotic expression of *unc-45* is sufficient for normal muscle structure (Venolia and Waterston 1990), what is the role of maternal UNC-45? If UNC-45 has a role in the early embryo, that role would only have become apparent in embryos sufficiently depleted of UNC-45; that is, in those embryos resulting from a mother homozygous for an *unc-45* null allele. Since the strongest alleles of *unc-45* are zygotic lethal at the two-fold stage of embryogenesis (Pat phenotype), these adults do not normally exist and such a function would not have been apparent from previous analysis (Venolia and Waterston 1990).

To genetically separate the possible roles of UNC-45 in the adult muscles from the germline and early embryo, we relied on both the muscle rescuing ability and the apparent lack of germline expression of the pDP#WA036 transgenic construct as described above. Transgenic animals homozygous for the temperature-sensitive (ts) *unc-45* allele *r450* are viable and fertile at the restrictive temperature (as described previously, Venolia *et al.* 1999). Transgenic animals homozygous for the *unc-45* lethal allele *st601* were somatically rescued for movement, but sterile, with a visibly abnormal germline (not shown). No GFP expression was seen in the germline of the transgenic lines, but we assume that the *r450* ts allele at the restrictive temperature provides at least some germline function sufficient for fertility, consistent with the provision of at least some somatic muscle function in *r450* ts homozygotes (Ao and Pilgrim 2000). This leads us to postulate that UNC-45 has an essential germline function which is lacking in the transgenic *unc-45(st601)* homozygous animals as well as in RNAi treated animals (below).

Neither the *r450* ts allele nor the *st601* lethal alleles show any dominant effects on early embryogenesis (not shown). We next examined animals heteroallelic for the ts and lethal alleles, carrying the pDP#WA036 transgene. The intent was to produce viable and fertile adult animals, but with a reduced contribution of UNC-45 function provided to the embryos. *r450/st601* genotype in the absence of the transgene is lethal at the two-fold stage. At all temperatures, the transgenic animals showed near normal movement and fertility. In growth at the restrictive temperature, animals had ameliorated germline abnormalities seen in transgenic animals homozygous for the lethal allele and fertilization took place, but no viable progeny resulted. Instead, the temperature-depleted embryos produced from these somatically rescued heteroallelic adults showed defects much earlier than the previously reported 'Pat' phenotype. Embryos depleted for maternal UNC-45 exhibit several defects, most obviously, frequent failure to complete cytokinesis, while nuclear division proceeds, often resulting in apparently polyploid daughter cells (Figure 2.2). The embryos depleted for UNC-45 may have initially established A/P polarity (based on the attempt to form cleavage furrows in asymmetrical positions along the embryo), but the most severely affected embryos failed to complete a single cytokinesis. Typically, embryos attempt cytokinesis with varying degrees of furrow ingression; few if any successfully complete cytokinesis on the first attempt (0/8 embryos examined by time lapse). Following cytokinetic failure, the furrows subsequently regress. The embryo repeatedly attempts cytokinesis each time the nuclei divide (over at least one hour of observation) and on these attempts, the furrow would only occasionally complete (3/8 embryos examined). Temperature depleted embryos that successfully complete one cytokinetic cleavage invariably fail in subsequent rounds, while nuclear division appears to continue. No viable embryos have been recovered out of hundreds examined. This effect is due to the presence of the *st601* nonsense allele in the mother as embryos produced at the restrictive temperature by homozygous *r450/r450* adults with or without the transgene do not exhibit cytokinetic defects.

We attempted to determine the role of UNC-45 in the early embryo using RNA-mediated interference. As reported previously (Venolia *et al.* 1999), embryos resulting from broods laid by adults 7 to 8 hours after injection of a dsRNA corresponding to part of the *unc-45* cDNA showed phenotypes consistent with arrest at the two fold stage of embryogenesis. Adults treated with RNAi for longer times (+45hrs post injection) inevitably become sterile. Sterility due to *unc-45* knockdown in a subset of animals following RNAi treatment has also recently been reported by others, who examined *unc-45* as part of conducting a genome wide screen (Kamath *et al.* 2003). We re-examined the broods of *unc-45* RNAi injected animals, concentrating our attention on the last eggs laid before sterility results. We were able to identify embryos from each brood that showed very little furrow invagination and a more severe defect than the temperature depleted animals, consistent with what we see for the temperature depletion of *unc-45*. Therefore, maternally provided UNC-45 appears to be essential and has a previously unrecognized role in embryonic cytokinesis.

2.3.3 UNC-45 interacts with non-muscle myosins

UNC-45 co-localizes with MHC B in the body wall muscle, and the yeast homologue Rng3 interacts with myosin in vivo. Therefore, we attempted to identify other myosins that may interact with UNC-45 in vivo using the yeast two-hybrid method (Fields and Sternglanz 1994; Phizicky and Fields 1995; Gietz and Schiestl 1995; James *et al.* 1996) to screen a *C. elegans* cDNA library using the full length *unc-45* cDNA as a bait. Several weak and three strong positives were identified among 10^6 transformants examined. Purifying the plasmid DNAs and re-transforming them into yeast confirmed the positive interactions of UNC-45 with the preys. As a control, bait plasmids that encode the SNF1 fusion protein (Durfee, *et al.* 1993), and the FEM-2 protein phosphatase (Pilgrim *et al.* 1995) were tested. SNF1 displayed a positive interaction with SNF4 protein in the yeast assays as expected, but neither SNF1 nor FEM-2 interacted with the putative UNC-45-dependent positives as judged by lack of growth on appropriate selective media. After sequencing the cDNA fragments and cloning into a different 'prey' vector backbone (pGAD, James *et al.* 1996), their interactions with UNC-45 were reassayed in yeast, and the same results were obtained.

Of the strong positives (Figure 2.3), two encode non-muscle myosin proteins: one is a fragment of *nmy-2*, and the other one is a fragment of *hum-2*, a class V myosin (Baker and Titus 1997) in both cases the fragments correlate to regions within the head domain of the myosins. Class V myosins may act as vesicular motors (Titus 1997). The third positive was identified as UFD2, however further analysis was not pursued. UNC-45 has two regions of sequence similarity to other proteins: the TPR domain at the amino terminus, and the UCS domain at the carboxyl terminus. The UCS family members (CRO1, She4p and Rng3p) all lack the TPR domain, suggesting a specific myosin-binding activity may be reflected in the conserved similarity, and the portion of UNC-45 C-terminus to the TPR domain has been reported to bind myosin in vitro (Barral *et al.* 2002). To address this with respect to HUM-2 and NMY-2, we tested a subclone of UNC-45 containing solely the TPR domain (amino acids 1-112). Neither HUM-2 nor NMY-2 showed significant interaction with bait containing just the TPR domain of UNC-45. Therefore, the myosin-binding domain lies within the larger fragment of UNC-45 containing the UCS domain.

The isolated cDNA fragments of the two myosin molecules encode only a small part of the entire proteins, which are nearly 2000 amino acids in length. A 530 amino acid fragment of the NMY-2 head domain (amino acid 469 to 999) is sufficient for the two-hybrid interaction with UNC-45 (Figure 2.3). The fragment of *hum-2* corresponds to amino acids 540 to 680 in the head domain that ends downstream of the ATP binding site and begins about 20 amino acids upstream of the actin-binding site. Thus, the fragments of myosin isolated in each of the two cases overlap (Figure 2.3). This small region also overlaps with region of MHC B that is sufficient to localize UNC-45 to thick filaments in vivo, using MHC A/B chimeric molecules (P. Hoppe, W.A. and D.P., unpublished data; Hoppe and Waterston 1996).

In order to estimate the strength of the yeast two-hybrid interactions a quantitative β-galactosidase activity assay was performed. There is no significant difference in level of reporter gene activity with NMY-2 than HUM-2 (Figure 2.3B) when full length UNC-45 is expressed as bait. However, the TPR domain alone shows minimal interaction with either myosin as shown by the lack of growth and low β-gal activity. Therefore, we have shown that NMY-2 interacts specifically with UNC-45.

2.3.4 UNC-45 co-localizes with NMY-2 in the early embryo

For the two-hybrid interaction to be meaningful, it is necessary to show that the two proteins are at least expressed in the same cells at the same time. While the expression pattern of HUM-2 has not been reported, a strain homozygous for a putative null allele *(ok596)* is viable, fertile and moves normally, suggesting that the embryonic lethality of *unc-45* is unlikely to result through effects on HUM-2. Both maternal UNC-45 and NMY-2 proteins are present in the early embryo; NMY-2 was initially identified as a non-muscle myosin II heavy chain that interacts with the C-terminus of PAR-1, a putative kinase (Guo and Kemphues 1996). NMY-2 is necessary in the embryo for normal asymmetric localization of PAR-1, -2 and -3 (Guo and Kemphues 1996). Furthermore, NMY-2 is localized at the embryonic cleavage furrow of the two-cell embryo and required for polarized cytoplasmic flow and cytokinesis (Guo and Kemphues, 1996; Shelton *et al.* 1999). A non-muscle myosin regulatory light chain has also been implicated in this process (Shelton *et al.* 1999). The staining seen for UNC-45 (Figure 2.1C, D) matches the staining reported for NMY-2 (Schumacher *et al.* 1998; Shelton *et al.* 1999), so we examined the localization of both proteins in the same embryos. We examined embryos labeled with NMY-2 and UNC-45 antibodies under regular and confocal microscopy. Figure 2.4 (A-F) shows images of 2-cell and ~20-cell stages of wild-type embryos, demonstrating that NMY-2 and UNC-45 are indeed concentrated at the cell cortex, and the staining patterns are largely coincident (although there are slight differences in intensity). Cortex staining for UNC-45 is apparent both where a fluorescent secondary antibody is used, and where UNC-45 antisera is directly labeled with a fluor (data not shown). Therefore, the two proteins co-localize in vivo, supporting the two-hybrid evidence that they may physically interact.

It remains to be determined whether this interaction is productive *in vivo*. However, we have shown that removal of MHC B protein from the thick filament results in lack of subcellular localization of UNC-45 staining (Ao and Pilgrim 2000), whereas wild-type UNC-45 is not necessary for at least partial localization of MHC B to the thick filament. RNAi directed against *nmy-2* results in embryos defective in asymmetric cell division and cytokinesis (Guo and Kemphues 1996; Shelton *et al.* 1999). In Figure 2.4G and H, the UNC-45 staining at the cortex of the cell is disrupted in *nmy-2(RNAi)* treated embryos, indicating that the localization of UNC-45 at the cleavage furrow is dependent on NMY-2. Thus, UNC-45 has an embryonic localization that is NMY-2 dependent, and NMY-2 is similar to MHC B, in that removal of the protein results in a change in subcellular localization of UNC-45. We examined NMY-2 immunostaining in a temperature depleted *unc-45* background (*st601/r450;edEx160*). The localization of NMY-2 remained cortical and no differences were detected from wild-type control embryos (shown in Figure 3.1). This result is expected from the minimal cytokinetic furrow activity indicating residual function for the UNC-45 maternal contribution of the *r450* allele.

2.4 Discussion

Here we provide evidence for an UNC-45 interaction with NMY-2, a non-muscle myosin, and a biological role for the UNC-45/NMY-2 interaction during embryonic cytokinesis. Previously, the evidence that UNC-45 or its mRNA is contributed maternally was indirect, as the lethal alleles of *unc-45* show maternal effects (Venolia and Waterston 1990). All reported phenotypes of *unc-45* mutants could be zygotically rescued, although the precise nature of the defect that leads to the lethality had not been adequately explained (Venolia and Waterston 1990). Reporter transgenes in *C. elegans* are poorly expressed in the germline (Seydoux *et al.* 1996; Seydoux and Dunn 1997; Kelly and Fire 1998), and any germline expression or maternal contribution of *unc-45* product would have been overlooked by the use of a GFP reporter. *In situ* hybridization to whole animals using *unc-45* cDNA as a probe suggests that *unc-45* transcripts are widespread in the early embryo as well as being strongly expressed in the adult hermaphrodite germline. There has not previously been an explanation for the detection of widespread early embryonic *unc-45* transcripts, as body wall muscle and pharynx muscle components are synthesized from zygotically transcribed genes. We show here that both *unc-45* mRNA and the protein are maternally provided. Since the antisera detects both maternal and zygotic UNC-45, the simplest explanation for the differences that we see between the immunolocalization and the reporter gene expression is that maternal UNC-45 is segregated to all cells in the embryo, and persists until the bean stage, at which point zygotic UNC-45 is synthesized only in muscle cells. In this way, zygotic UNC-45 synthesis mirrors that of other muscle thick filament components (Moerman and Fire 1997), but the existence of a maternal contribution among this set of genes would be unique.

Why is UNC-45 mRNA and protein maternally provided to the embryo if it is not required until later in zygotic development? The localization of UNC-45 staining to the cell cortex is not unique, as several maternally contributed components show cortical localization in the two-cell embryo (Rose and Kemphues 1998). Non-muscle myosin II NMY-2 (Guo and Kemphues 1996) is also enriched in the germline cells of the adult and at the cortex of early embryos. The observations that UNC-45 and NMY-2 co-localize in the embryo, that UNC-45 interacts with a fragment of NMY-2 in the yeast two-hybrid assay, and that UNC-45 localization in the embryo is disrupted if NMY-2 is removed using RNAi suggest a specific function for UNC-45 at this stage. We have now been able to define a precise role for maternally provided UNC-45 during embryonic cytokinesis. NMY-2 has been shown to be required for cytokinesis (Guo and Kemphues 1996) and a non-muscle myosin regulatory light chain, MLC-4, is also involved in this process (Shelton *et al.* 1999). Thus, this is the second instance of UNC-45 subcellular localization being dependent on a type II myosin molecule, potentially acting in a similar manner to aid in the assembly or stability of a myosin molecule during cytokinesis.

The co-localization of UNC-45 with body wall muscle thick filaments in an MHC B dependent manner suggested, but did not prove, that the interaction between UNC-45 and MHC B was direct. Our observation from this work strengthens that inference, as do *in vitro* studies (Barral *et al.* 2002). As mentioned above, both UNC-45 and NMY-2 are expressed in the germline cells of the gonad as well as the pre-morphogenesis embryos, and the localization of maternal UNC-45 at the cell boundaries is dependent on the presence of NMY-2. As well, UNC-45 and NMY-2 depletion both affect the same process of embryonic cytokinesis, further strengthening the idea that the UNC-45/NMY-2 interaction is biologically relevant. We found that polarity is established and maintained in UNC-45 heteroallelic temperature-depleted embryos indicated by an asymmetrically placed cleavage furrow suggesting either that the residual function of UNC-45 is sufficient to allow for polarity, or that NMY-2 does not require UNC-45 for establishment of the anterior-posterior axis. Consistent with residual UNC-45 function no defect in NMY-2 localization was observed for the temperature depleted embryos and NMY-2 remained cortical.

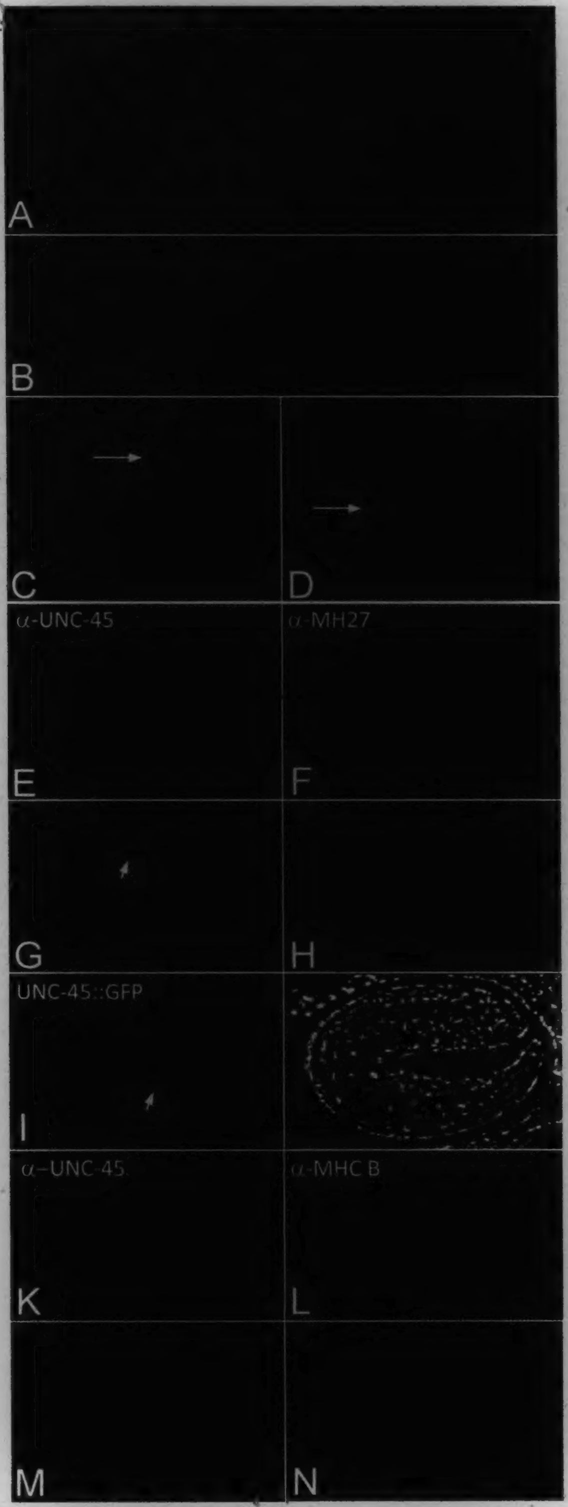
The interaction of UNC-45 with HUM-2 in the two-hybrid screen, while intriguing, has not been confirmed using an independent assay. As well, until it is shown that the expression patterns of UNC-45 and HUM-2 overlap, we can only state that the interaction is possible *in vivo*. This may be somewhat surprising since MHC B and NMY-2 are type II myosins, while HUM-2 is Type V (Baker and Titus 1997). These non-muscle myosin classes are largely differentiated based on similarity of the sequences of their head domains as well as the length of the rod domains (Titus 1997; Baker and Titus 1997). It has been recently reported that a member of the UCS family of proteins in budding yeast, She4p, can interact with a type V myosin as well as a type I myosin

(Wesche *et al.* 2003). However, there is striking sequence similarity in the regions of the head domain that is in common between all three myosins. We assume that the biochemical role that UNC-45 has in its interaction with MHC B in the body wall muscle is related to its interaction with NMY-2, and perhaps to HUM-2. Although UNC-45 has demonstrated chaperone activity *in vitro* (Barral *et al.* 2002), no chaperone activity has been shown *in vivo* although such an activity is consistent with the mutant phenotype. Since the role of non-muscle myosin II, particularly in cytokinesis, is a dynamic one where there is constant remodeling of the cytoskeleton, it may certainly involve chaperones and associated factors necessary for targeting and folding of myosin molecules (Bresnick 1999; Young *et al.* 2003). Rng3, which has sequence similarity to UNC-45 in fission yeast, is also involved in cytokinesis and has been shown to genetically interact with a type II myosin (Wong *et al.* 2000). Therefore, this may be an evolutionarily conserved process.

Since UNC-45 clearly acts through myosin in other tissues, it seems most likely that its role in the embryo is mediated (at least in part) through NMY-2. Therefore, unless there is evidence for a completely independent role for UNC-45, the simplest explanation is that UNC-45 is provided to assist NMY-2 in proper assembly into some higher order complex, to stabilize assembled myosin molecules and/or to localize NMY-2 to the proper subcellular location. Indeed, such a role may not be limited to embryos as a cytokinetic mechanism is necessary for proper oogenesis; transgenic *unc-45* null hermaphrodites are sterile due to a failure to produce oocytes (T. K. and D.P., unpublished data). Similarly, the F1 progeny of *nmy-2* RNAi treated animals that escape the embryonic lethality often develop to become sterile adults (Kamath *et al.* 2003) suggesting that NMY-2 may also be required in germline development, perhaps in a mechanism which also involves UNC-45. The UNC-45 homologue CRO1 is necessary for transition from syncytial to cellular growth in *Podospora anserina* (Berteaux-Lecellier *et al.* 1998), a process analogous to germ cell development in the *C. elegans* gonad (Schedl 1997) and *C. elegans* UNC-45 may play a similar role in many different tissues.

2.5 Figures

Figure 2.1. UNC-45 expression in the adult germline and early embryo. A) A hermaphrodite gonad doubly-stained with anti-UNC-45 antibody (green) and DAPI (blue), which stains the cell nuclei. B) Distal part of hermaphrodite gonad doubly stained with pre-immune serum and DAPI as controls. C and D) Two cell and ~16 cell embryos stained with anti-UNC-45. Arrows indicate concentration of UNC-45 at cell boundaries. In panels E-H, embryos are doubly stained for UNC-45 (E, G) and monoclonal MH27 (F, H), which recognizes an epitope associated with adherent junctions at the boundaries of hypodermal cells (Francis and Waterston 1985; Hresko *et al.* 1994). As the embryo begins to elongate, the UNC-45 staining is progressively restricted to the presumptive muscle cells (G, arrowhead). I and J) GFP and DIC images of a two-fold embryo transgenic for an UNC-45::GFP fusion protein, showing expression in the body wall muscles. K to N) UNC-45 staining is detectable throughout embryogenesis whereas MHC-B staining is only detectable in the developing body wall muscles. K and M are stained with anti-UNC-45, L and N with anti-MHC B (5-8). K and L) The same embryo at about 250 minutes after fertilization. M and N) The same embryo at the 1.5-fold stage of elongation. The images of L and N were exposed for the same time, serving as controls to show that while MHC B does not show staining in the early embryos, UNC-45 does.


A
B
C
D
α-UNC-45
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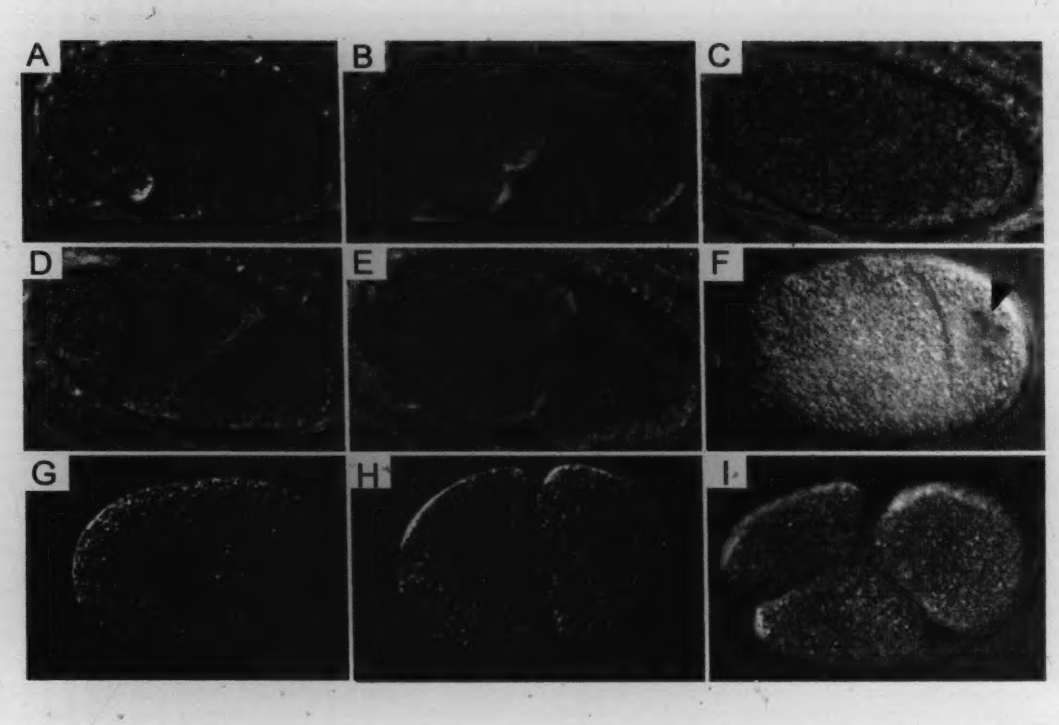
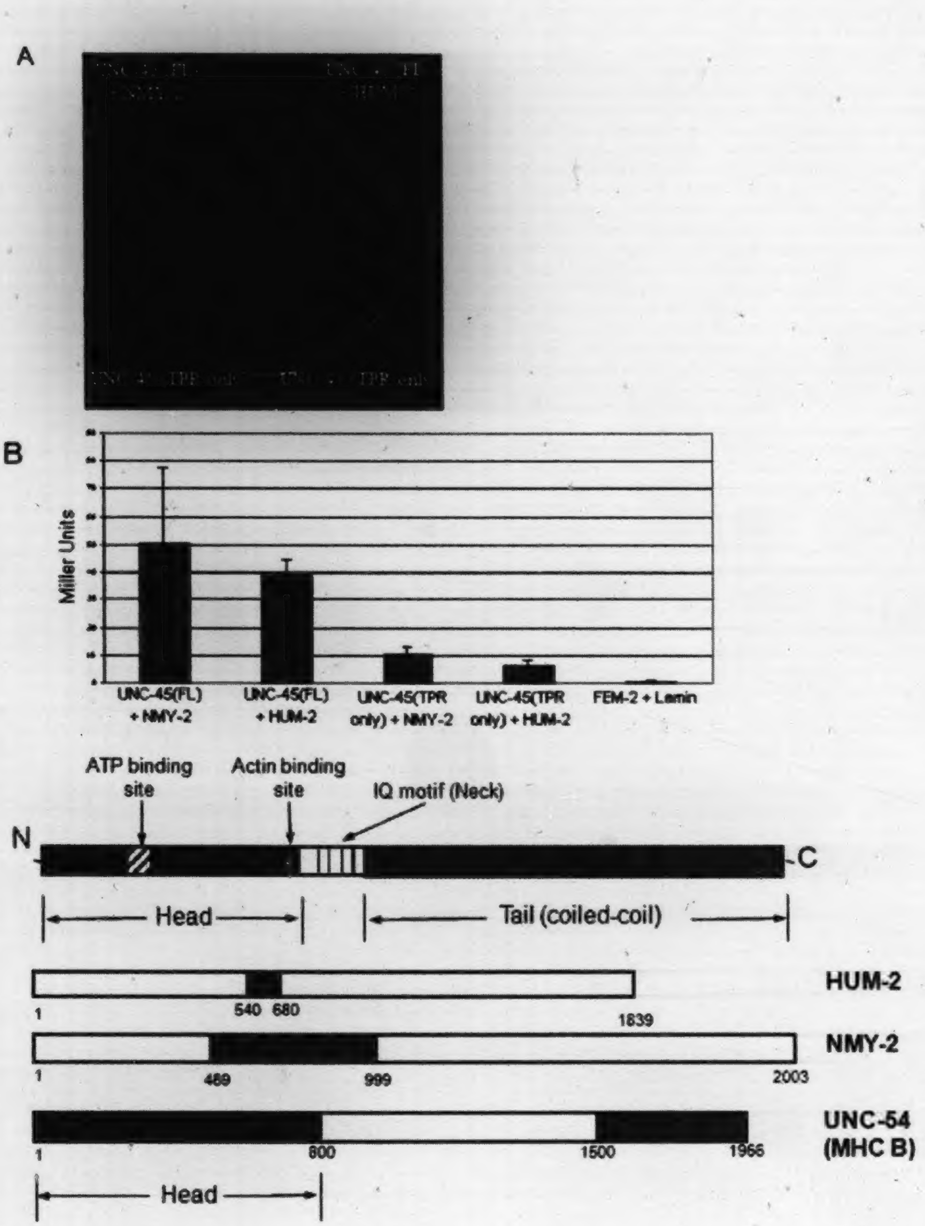


Figure 2.2. Cytokinesis defects in *C. elegans* embryos. Embryos were derived from mothers of the genotype *unc-45(st601let)/unc-45(r450ts)* carrying an extrachromosomal array with the wild-type *unc-45* cDNA fused to GFP, raised at 25°C. A-E) Cytokinesis defects in embryos derived from mothers of the genotype *unc-45(st601let) / unc-45(r450ts)* carrying an extrachromosomal array with the wildtype *unc-45* cDNA fused to GFP, raised at 25°C. Time course shows the same embryo photographed at different times. Complete cytokinesis was not observed, although many attempts were made. Times are relative to the beginning of observation. A) 0 minutes B) 15 minutes C) 25 minutes D) 40 minutes E) 70 minutes F) Embryo from a different mother of the same genotype. Notice the multiple nuclei in one cell (arrowhead). G-I) A wild type embryo is normally able to complete at least two cell divisions in a similar time. G) 0 minutes. Pronuclear meeting. H) 4 minutes. First cleavage. I) 14 minutes. Second cleavage. Embryos were observed under Differential Interference Contrast optics.

Figure 2.3. Characterization of isolated clones from a yeast two-hybrid screening of a *C. elegans* cDNA library with *unc-45* cDNA. A) Two of the clones identified from yeast two-hybrid screens. UNC-45 from a full length cDNA (pDP#WA036) interacts with the products of fragments of two different myosin cDNAs, NMY-2 and HUM-2. The TPR domain alone was tested and showed no interaction with either NMY-2 or HUM-2. B) Quantitative β-gal assay for the yeast two hybrid clones. C) Diagram of a conventional myosin II molecule, showing the ATP and actin binding sites in the head domain, IQ motif (light chain and calmodulin binding sequence) in the neck domain and the coiled-coil tail domain. Schematic diagram of MHC B and two myosins identified from the yeast two-hybrid screens that interact with UNC-45. HUM-2 is a type V myosin and the identified cDNA fragment encodes a 140 amino acid segment located in the head domain (grey box). NMY-2 is a non-muscle type II myosin and a region of 530 amino acids (grey box) located mostly in the head domain interacts with UNC-45. Note that these two identified regions overlap in the highly conserved head domain as indicated. In UNC-54, the portion of the molecule that is sufficient to localize UNC-45 to the body wall muscle thick filaments is indicated by the grey box. Detailed domain analysis of NMY-2 is not available.


A
UNC-45(FL)
+ NMY-2
UNC-45(FL)
+ HUM-2
UNC-45(TPR only)
UNC-45(TPR only)
B
Miller Units
UNC-45(FL) + NMY-2
UNC-45(FL) + HUM-2
UNC-45(TPR only) + NMY-2
UNC-45(TPR only) + HUM-2
FEM-2 + Lamin
C
ATP binding site
Actin binding site
IQ motif (Neck)
N
C
Head
Tail (coiled-coil)
HUM-2
1
540
680
1839
NMY-2
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489
999
2003
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1
800
1500
1966
Head

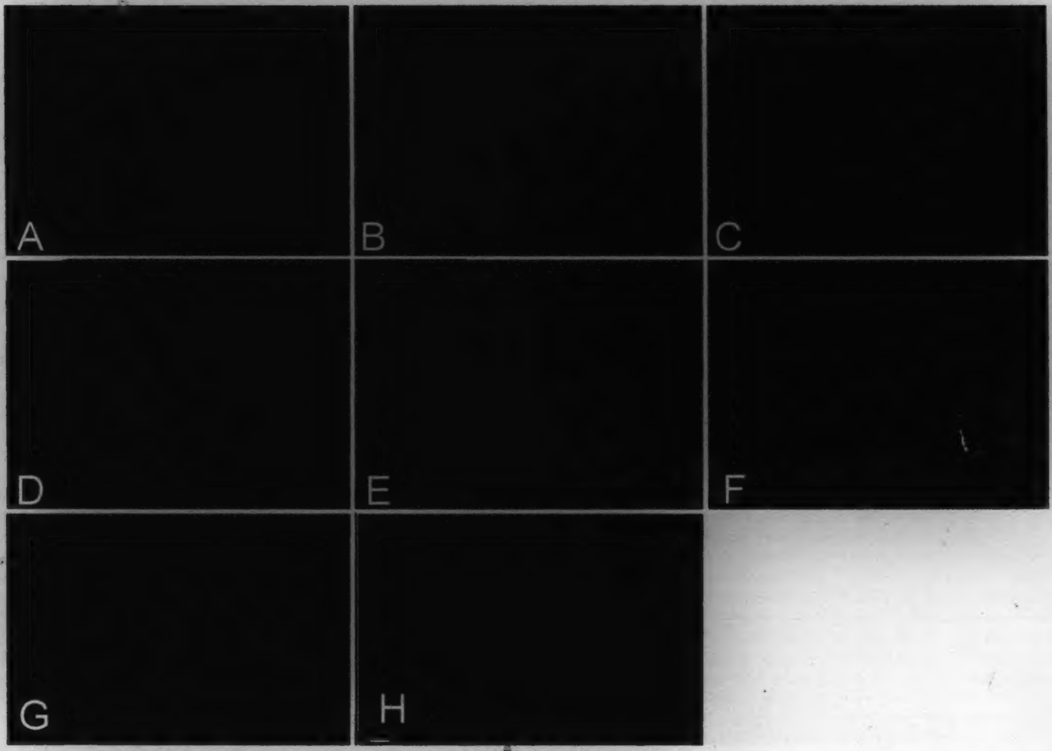

Figure 2.4. Immunolocalization of UNC-45 and NMY-2 in wildtype and *nmy-2(RNAi)* embryos. A-F) Wildtype embryos of increasing age stained with anti-UNC-45 (green) and anti-NMY-2 (red) to show the concentration of UNC-45 and NMY-2 at the cell boundaries. C) Merge of 2-cell embryos stained for anti-UNC-45 (A) and anti-NMY-2 (B) showing their co-localization. D-F) anti-UNC-45 (green) and anti-NMY-2 (red) staining in a ~20 cell stage embryo with merge. Images G and H) are embryos treated with RNAi against NMY-2, stained with anti-UNC-45 showing that the concentration of UNC-45 at the cell boundaries is disrupted with NMY-2 is knocked down.

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2.7 Appendix

Genotype	**Conditions**	**Phenotype**
unc-45(st601let)/unc-45(st601let); edEx160	Permissive temperature (15°C)	Sterile
unc-45(r450ts)/unc-45(st601let); edEx160	Permissive temperature (15°C)	Wildtype
	Restrictive temperature (25°C)	Embryonic lethal (Cytokinesis defect)
unc-45(st601let)/unc-45(st601let)	Permissive temperature (15°C)	Embryonic lethal (two-fold arrest)

Table 2.1. Genotypes of strains used in this study. *unc-45(st601)* is a null allele, *unc-45(r450)* is a temperature sensitive allele and *edEx160* is an UNC-45::GFP extrachromosomal transgenic rescue array with the *unc-45* promoter. Strains were raised at either the permissive or restrictive temperature for at least 24 hrs prior to phenotypic analysis.

3. UNC-45 is required for NMY-2 contractile function in early embryonic polarity establishment and germline cellularization in *C. elegans.*[2]

3.1 Introduction

Molecular chaperones are required in many different cell contexts to ensure the proper folding and/or maintenance of a vast array of proteins essential for cell survival. We have shown that UNC-45 interacts with a maternally contributed type II non-muscle myosin (NMY-2) and NMY-2 is required for the proper localization of UNC-45 to the cell cortex in the *C. elegans* embryo (Kachur *et al.* 2004). NMY-2 is necessary for cortical contractility that is required for the establishment of embryonic polarity, as depletion results in symmetrical early cleavages (Guo and Kemphues 1996). Local weakening of posterior myosin activity is thought to drive the retraction of the microfilament cortex away from the sperm entry point (Munro *et al.* 2004; Jenkins *et al.* 2006). NMY-2 is also required in the embryo for polar body extrusion, cytokinesis and polarized cytoplasmic flows (Cuenca *et al.* 2003).

Since UNC-45 and NMY-2 are involved together during cytokinesis, (Kachur *et al.* 2004); and NMY-2 is needed for other embryonic processes, UNC-45 may also be involved in embryonic contractility and polarity establishment. The major components of the contractile apparatus, actins and myosins, have not been directly tested for their role during germ cell development. NMY-2 appears to be required for hermaphrodite fertility as depletion of NMY-2 by RNAi results in sterility (Simmer *et al.* 2003; Piekny *et al.* 2003; Piano *et al.* 2002; Kamath and Ahringer 2003); but the cause of this sterility has not been fully investigated.

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[2] *A version of this chapter has been published.*

Kachur, T. Audhya, A and Pilgrim, D. 2008. Developmental Biology. 314(2):287-99. Anjon Audhya constructed the OD50 strain.

We show here that UNC-45 does indeed function during early embryogenesis for processes other than cytokinesis as well as germline cellularization. Examination of *unc-45(RNAi)* embryos has exposed roles for maternal UNC-45 beyond cytokinesis, including roles in myosin function that are required for proper A/P polarity establishment, polar body extrusion and cortical flows. We show that UNC-45 and its partner, NMY-2, are required for the initiation and completion of oocyte cellularization furrows.

Additionally, we show that the male gonad undergoes the same form of cellularization of syncytial cells to produce sperm as hermaphrodite oocyte development. Contrasting the model that UNC-45 is responsible for generalized folding of the myosin head, we show that myosin binds actin in the absence of UNC-45. This suggests that UNC-45 is not required for the folding or maintenance of the actin binding domain of NMY-2 but it does play a role in the overall contractile ability of myosin once the head is bound to actin. Our results indicate that UNC-45 is essential to all processes where NMY-2 has been implicated, suggesting that UNC-45 is the only myosin II chaperone that regulates myosin function during early development.

3.2 Materials and Methods

3.2.1 Nematode cultures and strains

C. elegans strain N2 Bristol (wild type) was obtained from the stock collection of the MRC Laboratory of Molecular Biology, Cambridge, UK and maintained as previously described (Wood 1988). The alleles of *unc-45* have been described previously (Venolia and Waterston 1990; Barral *et al.* 1998; Ao and Pilgrim 2000). Temperature depleted hermaphrodites (DP398) consist of lethal (*st601*)/temperature sensitive (*r450*) heteroallelic animals somatically rescued with an extrachromosomal array (*edEx160*) as previously described (Kachur *et al.* 2004). Sterile animals (DP397) are homozygous for the lethal allele and somatically rescued for *unc-45* (*st601/st601*; *edEx160*), but not rescued in the germline because transgenic arrays are often silenced in the germline (Kelly and Fire 1998). The NMY-2::GFP (*zuIs45*) strain was kindly provided by E. Munro (Munro *et al.* 2004).

To visualize the membrane partitions that form between developing germ cells in the gonad, we expressed a PH domain that specifically recognizes phosphatidylinositol 4,5-bisphosphatase generated on the plasma membrane fused to a re-engineered form of mCherry, whose sequence now reflects a *C. elegans* codon bias. A plasmid encoding mCherry:PH under the control of PIE-1 regulatory sequences was generated by cloning the PH domain of rat PLC1δ1 into the SpeI site of pAA64. This construct (*ltIs44*) was then stably integrated using biolistic bombardment (Praitis *et al.* 2001). The DP399 strain contains a lethal allele of *unc-45 (st601)* over a *dpy-1* marked *sC1* (*unc-*

45(st601)+/sC1(s2073)[dpy-1(s2170) +]; *zuIs45; edEx160).* The genotype of DP400 is similar to DP399, with the addition of *ltIs44* and DP401 strain genotype is (*unc-45(st601)/sC1*(*dpy-1(s2170)*)); *ltIs44; edEx160*).

3.2.2 RNA-mediated interference (RNAi)

RNAi (Fire *et al.* 1998) was performed by microinjection of dsRNA into L4 or young adult hermaphrodite gonads. Previously reported RNAi results (Kachur *et al.* 2004) showed a weaker phenotype because only the first embryos produced were examined. In the current work, embryos from *unc-45* dsRNA injected hermaphrodites were observed 38-48 hrs post-injection. Two different double stranded RNAs (dsRNA) corresponding to parts of the mRNA encoding either the central (509 bp fragment) or C-terminal portions (644 bp fragment) of the UNC-45 protein were generated using a MEGAscript T7 in vitro transcription kit (Ambion). Both dsRNAs used resulted in similar phenotypes and a range of concentrations of dsRNA were equally effective. Adult hermaphrodites treated with dsRNA were dissected on 3% agarose pads and live image recordings of embryos were taken.

Cytoplasmic granule movement speeds were determined by visual inspection of individual granules in the center of the embryo over one minute using an ocular micrometer. Using this method, we were able to replicate wildtype granule flow rates previously measured (Shelton *et al.* 1999).

Three classes of embryos were defined as mild, moderate or severe using similar classifications detailed for *nmy-2(RNAi)* (Cuenca *et al.* 2003). The various severities were obtained by differing recovery times after incubation. For example, mild phenotypes were often observed 36-38 hrs post injection while severe phenotypes were observed up to 48 hrs post injection.

nmy-2(RNAi) was completed by injection of an 1182 bp fragment of dsRNA into young N2 adult hermaphrodites. Sterility in the F1 generation was observed 84 hrs post injection.

For visualization of sterile gonads *in vivo*, adult worms were anaesthetized in M9 with 0.1% tricaine and 0.01% levamisole and mounted on 3% agarose pads.

3.2.3 Fluorescent visualization and microscopy

NMY-2::GFP sterile animals required gonad dissection because of the competing GFP fluorescence in the *edEx160* rescue construct. Gonads were dissected in 1 x PBS and mounted on 3% agarose pads.

Staining of DNA and actin was completed by dissecting hermaphrodites in 1x sperm salts (50 mM PIPES, 25 mM KCl, 1 mM MgSO4, 45 mM NaCl, 2 mM CaCl2) (Shaham, 2006) on poly-L-lysine slides (Erie Scientific) then fixed in 3% paraformaldehyde. Gonads were permeabilized by freeze cracking, immersed in 95% ethanol for 1 min and washed in 0.1% PBS-Tween. Nuclei were visualized by adding 2.5 µL of 100 µM DAPI (Sigma) and 0.02 units Alexa-546 labeled phalloidin to the specimens (Molecular Probes). NMY-2::GFP signal was maintained after the fixation procedure and GFP was visualized without antibody addition. Images were taken using Retiga EXi camera mounted on a Zeiss Axioscope 2 fitted with a 63 x 1.4 N.A. lens and a mercury lamp. Images were taken in black and white and false colored using Graphic Converter software.

3.2.4 Blebbistatin treatment

100 mM blebbistatin (Straight *et al.* 2003) diluted in 95% ethanol was co-injected with Alexa-546 phalloidin into the distal gonad of adult hermaphrodites then injected worms were incubated for 20 minutes prior to microscopy. Control injections were performed with Alexa-546 phalloidin diluted in 95% ethanol.

3.3 Results

3.3.1 Myosin dynamics in an *unc-45(RNAi)* embryo.

UNC-45 is expressed in the *C. elegans* germline and early embryo, despite lack of corresponding muscle myosin expression (Kachur *et al.* 2004). We have previously shown that UNC-45 can physically interact with NMY-2 and that the localization of UNC-45 to the cell cortex requires functional NMY-2 (Kachur *et al.* 2004). To test the model that UNC-45 is required for all processes requiring NMY-2 function, we examined myosin cortical dynamics in the one-cell embryo by observing the *in vivo* localization of NMY-2::GFP (Munro *et al.* 2004) in an *unc-45(RNAi)* embryo (Figure 3.1).

After fertilization in wildtype, downregulation of myosin activity in the posterior zygote causes asymmetric myosin activity and formation of large interconnected myosin foci that contract into visible ruffles in the anterior embryonic cortex (Figure 3.1A). Eventually the myosin retracts to form an anterior cap delineating anterior and posterior domains (Munro *et al.* 2004) (Figure 3.1B). The polarity signals are transduced such that the sperm and egg pronuclei meet near the posterior of the cell (Figure 3.1C) and migrate back to the centre prior to mitosis. After anterior retraction, the myosin then accumulates at the cytokinetic furrow (Figure 3.1F) where the diameter of the actomyosin ring decreases to complete cytokinesis (Figure 3.1H). In all *unc-45(RNAi)* embryos cortical contractility was reduced to varying degrees that could be classified into three distinct categories. The three classes displayed similar defects including failed polarity establishment as shown by a symmetrical pronuclear meeting location (47% of egg length) and reduced cortical granule flow (Table 3.1). We assume that these three categories reflect different effectiveness of RNAi. In *unc-45(RNAi)* mildly affected embryos cortical ruffling was reduced during pronuclear migration (Figure 3.1I) and pseudocleavage was absent (Figure 3.1I). Occasionally, there appeared to be an asymmetric pronuclear meeting location but did not result in asymmetric cell division (Figure 3.1K). NMY-2::GFP did not gather into a contractile anterior domain (Figure 3.1J); instead, myosin remained at the cortex as smaller noncontractile punctae that were localized throughout the embryo (Figure 3.1J inset) despite partial clearing from the site of sperm entry in the posterior. The contractile activity present in the embryo was sufficient for proper polar body extrusion (Figure 3.1K asterisk). Cytokinesis was successful in mildly affected embryos but the furrow was placed at a more central location occurring at 48% as opposed to the wildtype placement of 42% of egg length (Table 3.1).

Moderately affected embryos exhibited phenotypes similar to the mildly affected embryos and some additional defects including failed polar body extrusion and cytokinesis (Figure 3.1M-T). NMY-2::GFP showed early cortical localization similar to mildly affected embryos with no formation of discrete cortical domains (Figure 3.1N inset and P). Cytokinesis was attempted at a symmetric location with the cytokinetic furrow partially ingressing (Figure 3.1Q arrow) then regressing (Figure 3.1S) but NMY-2::GFP concentrated at furrow invagination despite failed cytokinesis (Figure 3.1R). When the cleavage furrow was unsuccessful and regressed, NMY-2::GFP disappeared from the furrow location (Figure 3.1T). At spindle retraction, karyomeres were observed (13/14 of embryos examined) (Table 3.1) indicating a failure in polar body extrusion (Figure 3.1S arrow).

Severely affected embryos retained cortical localization of NMY-2::GFP but exhibited no contractile activity where defects observed were similar to moderately affected embryos but there was no attempt at furrow ingression during cytokinesis (Figure 3.1U-X). There was no large myosin foci formation in the early embryo during pronuclear migration (Figure 3.1V inset), indicating that the appearance of larger foci is dependent on cortical contractility. Polarization of the cortex did not occur (Figure 3.1X) despite some localized clearing away from the site of sperm entry. In an embryo that was fertilized at a lateral position equidistant from the poles (Figure 3.1Y-BB) there was a localized clearing of cortical myosin away from the site of sperm entry. Sperm entry caused myosin to disperse from the entry position (Figure 3.1Z asterisk) but at pronuclear meeting myosin failed to complete polarization to the anterior. This indicated that the polarity defects observed likely arose from unsuccessful contractility in the future anterior domain, and not from defects in the dissolution of contractile structures in the posterior, both of which are required for the polarization process. Although some UNC-45 activity must be present in order for the hermaphrodite to be fertile (see below) the severity of phenotype suggests that UNC-45 function is reduced to near-null activity, therefore we suggest that UNC-45 mediated myosin function is not required for localized clearing from the sperm entry site. Severely affected embryos fail cytokinesis entirely with no membrane invagination observed despite NMY-2::GFP localizing at the site of the presumptive furrow (Figure 3.1BB). These results indicate that UNC-45 is required for the production of functional myosin but myosin need not be contractile to localize to the cortex. Additionally, myosin function is required for anterior retraction but not dissolution of the microfilament network away from the site of sperm entry.

3.3.2 Localization of polarity determinants in an *unc-45(RNAi)* background

The unsuccessful cortical retraction of myosin to the anterior in *unc-45(RNAi)* embryos suggested that the source of the polarity defect lay upstream of the PAR proteins. The PAR proteins localize asymmetrically in the embryo and act to transduce the polarity signal into different cell fates. Microfilament cortical retraction concentrates the PAR-3/PAR-6/PKC complex in the anterior domain; (Etemad-Moghadam *et al.* 1995; Tabuse *et al.* 1998; Hung and Kemphues 1999; Schneider and Bowerman 2003; Nance 2005) thus permitting PAR-1/PAR-2 expansion in the posterior (Severson and Bowerman 2003; Cuenca *et al.* 2003). When NMY-2 is depleted, PAR-2::GFP fails to localize to the cortex and PAR-6::GFP remains cortical around the entire embryo (Cuenca *et al.* 2003). Similar results are obtained in all classes of *unc-45(RNAi)* embryos, as we see that PAR-2::GFP fails to localize to the cortex at pronuclear meeting with no posterior concentration resulting in diffuse cytoplasmic signals (Figure 3.2C and D). Normally PAR-2::GFP accumulates at the posterior cortex (Figure 3.2A and B). PAR-6::GFP fails to be restricted to the anterior at pronuclear meeting and remains cortical around the entire embryo (Figure 3.2G and H). Thus, we conclude that myosin contractility is required for PAR protein asymmetric localization and UNC-45 acts in conjunction with NMY-2 upstream of the PAR proteins.

3.3.3 UNC-45 and NMY-2 are required for oocyte cellularization

Our results so far indicate that UNC-45 is required for NMY-2 function in many different processes in the embryo but NMY-2 has also been shown to localize to the sites of oocyte cellularization (Piekny and Mains 2002; Figure 3.6A). Therefore we tested the necessity of both UNC-45 and NMY-2 during germline cellularization (Figure 3.3). In wildtype, the germline nuclei enter meiosis in the distal gonad where the syncytial nuclei are partially enclosed by membrane into primordial germ cells (PGC). As nuclei proceed around the turn into the proximal gonad the membranes begin to enclose the nuclei further until they are completely surrounded by membrane and are independent oocytes (Figure 3.3A schematic). Depletion of *nmy-2* by RNAi resulted in adults that did not show any cellularized oocytes in the proximal gonad (Figure 3.3B). Therefore we tested if myosin contractility is necessary for germline membrane ingressions and completion of cellularization. In order to visualize the germ cell membrane for defects in cellularization, we used an mCherry:PH reporter, a pleckstrin homology domain-containing membrane marker.

The progressive nature of cellularization in the gonad provides an alternative system for studying the molecular dynamics of UNC-45 and NMY-2. The membrane in the gonad normally forms a distinctive pattern that has been previously observed for actin where the membrane boundaries surround the syncytial nuclei (Figure 3.3C and D).

Hermaphrodites homozygous for a lethal allele (*st601*) were somatically rescued by the *unc-45* (+) extrachromosomal array (*edEx160*) to rescue the muscle defect but high copy transgenes are often silenced in the germline thus eliminating maternal contribution of UNC-45. The somatically rescued animals that lack UNC-45 in the germline (genotype *st601/st601; edEx160; ltIs44*) were completely sterile due to a severe disruption in membrane invagination and organization throughout the germline (Figure 3.3E and F). Similar degrees of membrane disorganization were observed for *nmy-2(RNAi)* (Figure 4.1H).

Using this mCherry:PH membrane marker it was possible to identify the earliest defects in germline cellularization. In the L4 larval stage, there was a uniform arrangement of membranes throughout the gonad (Figure 3.3G and H). In UNC-45 depleted L4 larvae there was an obvious disorganization of both the proximal and distal gonad membranes (Figure 3.3I and J). Occasionally, there appeared to be some attempts at cellularization in the extreme proximal region, but this is unlikely to reflect successful spermatogenesis (see below). In the wildtype L3 larvae the gonad extends distally away from the future position of the vulva but has not made the proximal to distal turn to return towards the centre of the animal. The L3 wildtype germline displayed a normal cellularization arrangement (Figure 3.3K and L). Once again when depleted for UNC-45, the germline membranes failed to surround the proliferating nuclei in the L3 larvae (Figure 3.3M and N). At the L2 stage, the gonad is composed of very few cells that begin to proliferate to generate the PGC nuclei and somatic gonad. In wildtype L2 all the visible germline nuclei in the small gonad were enclosed by membrane (Figure 3.3O and P). In UNC-45 depleted worms this cellularization still occurred and the germline exhibited an ordered membrane arrangement (Figure 3.3Q and R).

We show that the first identifiable defect in germline cellularization is at the L3 stage where germ cell nuclei begin to be separated from the developing soma. The necessity for UNC-45 for germ cell proliferation and development suggests that UNC-45 mediated myosin function is required for initiating cellularization of the mitotically proliferating nuclei as well as completion of cellularization in the proximal gonad. We conclude that UNC-45 is required for the formation of membrane barriers in cellularization where loss of myosin contractility results in the lack of organized membrane structures throughout the germline.

3.3.4 Germline cellularization defects in spermatogenesis

Sperm are formed for a short period of time late in the L4 larval stage of hermaphrodites and signals within the germline cause the differentiation of the most proximal nuclei of the syncytium into sperm. Therefore, we examined both hermaphrodite and male adults to see if the small regions of membrane organization that were observed in L4 larvae may represent attempts at spermatogenesis (Figure 3.4). In wildtype adult hermaphrodites the sperm are visible in the spermatheca using DIC optics (Figure 3.4A asterisk); however in hermaphrodites deficient for UNC-45 function sperm were not visible in the proximal gonad (Figure 3.4B arrow). Consistent with this, NMY-2::GFP (*zuIs45*) localized to the membranes of proximal PGCs in late L4 hermaphrodites where spermatogenesis is occurring (Figure 3.4C-E) and was present in a similar pattern in males (Appendix Figure 3.8).

The male germline consists of a single gonad arm that extends the length of the male and loops back to form the distal end of the gonad (Figure 3.4F). The male germline showed a pattern of germ cell membranes similar to that seen in the hermaphrodite (Figure 3.4G and H). In UNC-45 depleted males there was a large disorganization of the germline membrane (Figure 3.4I and J) consistent with the defect observed in hermaphrodites. In UNC-45-depleted males there was no accumulation of sperm in the spermatheca (Figure 3.4M and magnification in N) compared to wildtype males (Figure 3.4K and magnification in L) suggesting that UNC-45 is required for spermatogenesis. Our results indicate that sperm formation involves a myosin based apparatus for completion of spermatogenesis.

3.3.5 Nuclear proliferation and meiotic progression is defective in sterile animals

In both males and hermaphrodites depleted for UNC-45 the germline contained large nuclear structures that were not consistent with wildtype meiotic nuclei, suggesting that a consequence of loss of myosin function included meiotic defects. In UNC-45 sterile hermaphrodites, nuclear proliferation and meiotic progression were defective (Figure 3.5). Normally the gonad contains nuclei that leave the mitotic proliferating zone in the extreme distal gonad and progress through meiosis from the distal to the proximal gonad (Figure 3.5A). Wildtype nuclei are closely opposed to the interior face of the gonad and are organized into discrete PGCs.

unc-45 sterile animals (*st601/st601; zuIs45; edEx160*) showed abnormal nuclear morphology throughout the gonad (Figure 3.5B); the nuclei did not localize to the outer edge of the gonad and were not of uniform shape or size suggesting a meiotic defect. However, animals can be partially depleted for UNC-45 using a hemizygous temperature sensitive allele (*r450*) and raised at the restrictive temperature (*st601/r450; edEx160*). This reduction in UNC-45 still allows nuclei to progress normally through meiosis despite failed cellularization on several occasions as shown by dinucleated oocytes visible with nuclei arrested normally in meiotic diakinesis (Figure 3.5E and F).

3.3.6 Organization of the microfilament cytoskeleton in UNC-45 sterile adults

To test the function of UNC-45 on the NMY-2 contractile head domain more directly, we examined the co-localization of both actin and myosin in *unc-45* sterile hermaphrodites. When UNC-45 was depleted, the non-functional NMY-2::GFP did not organize into a defined pattern (Figure 3.6D) but still localized to the membrane in the gonad (Appendix Figure 3.9). Microfilaments normally arrange in a pattern similar to that observed for wildtype non-muscle myosin with repeating primordial germ cell boundaries in the distal region (Hird 1996) (Figure 3.6B). In the absence of UNC-45, both myosin (Figure 3.6D) and actin (Figure 3.6E) organization are disrupted.

Normally actin and myosin co-localize (Figure 3.6C) but UNC-45 is thought to aid in the folding of the myosin head, including the actin binding domain (Barral *et al.* 2002) and this would suggest that myosin cannot bind actin without UNC-45-mediated folding.

However, this result suggests that myosin remains capable of associating with actin in the absence of UNC-45 as shown by their co-localization in sterile gonads (Figure 3.6F, magnification in I (asterisk)). Although it is possible that some UNC-45 could be contributed from the soma, no UNC-45::GFP is observed in sterile gonads suggesting a near complete reduction in UNC-45 function in sterile gonads.

In order to confirm that the NMY-2- microfilament co-localization was dependent upon the binding of the myosin head to the actin filament, we treated the worms with blebbistatin to specifically disrupt the actin-myosin interaction. Blebbistatin is a potent myosin II inhibitor that blocks myosin in an actin detached state (Straight *et al.* 2003). Blebbistatin treatment caused NMY-2::GFP to dissociate from the germline cortex while the actin staining pattern remained largely unchanged (Figure 3.7A and B). The injection of blebbistatin did not result in severe cellularization defects because it is applied only over a short period of time. Treatment with solvent alone had no effect on myosin or actin localization (Figure 3.7C and D), indicating that the loss of localization of myosin was specific to the drug. Based on this result, we conclude that co-localization of NMY-2 with actin in germline membranes is dependent on its ability to physically interact with actin. Furthermore, our analyses of UNC-45 suggests that this chaperone-like protein is not required for the NMY-2-actin interaction, but rather plays a role in the contractile properties of the associated complex.

3.4 Discussion

UNC-45 is essential in all known type II myosin-based contractile activities in the germline and early embryo. The defect observed for *unc-45(RNAi)* embryos is a complete loss of microfilament-mediated cortical contractility as shown by lack of ruffling, cortical granule flow and pseudocleavage furrow formation culminating in the loss of anterior-posterior polarity establishment and failed cytokinesis. *unc-45(RNAi)* treated hermaphrodites eventually become sterile because the syncytial germline requires UNC-45 and NMY-2 to initiate the cellularization of nuclei into discrete germ cells. Our evidence suggests that UNC-45 reduction prevents myosin function although NMY-2 maintains actin binding activity. Therefore, UNC-45 is required for all NMY-2-mediated processes by ensuring the proper functioning of the NMY-2 head domain, excluding the actin binding domain.

Many type II myosins, like NMY-2, cannot be folded properly in various in vitro expression systems. Specifically, the initial folding of the head domain requires some sort of chaperone activity normally provided in vivo (Chow *et al.* 2002; Srikakulam and Winkelmann 2004). Our results show that UNC-45 is a chaperone required for acting on a subsection of the head domain because myosin can still bind actin in the absence of UNC-45 function; indicating that UNC-45 is required much later in the myosin head folding cascade than previously suggested and aids in folding of a specific region of the head or neck domain. UNC-45 must be acting in a different manner than its homologue in *S. pombe,* where mutants lacking Rng3p, a homologue of UNC-45 produce myosin that can no longer bind actin (Lord and Pollard 2004). A potential target of UNC-45 is the ATPase function of the myosin head that would account for the ability of myosin to bind actin but not contract, a process that requires the hydrolysis of ATP. The myosin head contains two distinct binding sites for actin or ATP and the cycling of ATP hydrolysis causes conformational changes that result in movement of the head domain (reviewed in Warrick and Spudich 1987). Loss of UNC-45 may attenuate myosin contraction by the misfolding of the ATPase domain causing rigor or tight association of myosin with actin without contraction. Thus, in the absence of UNC-45 myosin light chain could still associate with the neck of non-muscle myosin, allow dimerization of the tails and association at the cortex without undergoing contraction.

Functional myosin produced with the aid of UNC-45 is essential in the embryo for cortical flows and microfilament anterior retraction. The formation of contractile foci correlates to the ingressions seen with embryonic ruffling (Munro *et al.* 2004) and our results suggest that non-contractile myosin associates with the cortex as small punctae but must be functional to aggregate into foci. Polarization of the microfilament cytoskeleton in the early embryo occurs in two discrete steps with sperm entry causing dissolution of contractile structures in the posterior which, in turn, causes the local weakening of the cortex allowing retraction to the anterior. The polarity cue that causes the disappearance of myosin foci is a Rho-GTPase activating protein that negatively regulates myosin light chain function in the posterior (Jenkins *et al.* 2006). Activation of regulatory myosin light chain by phosphorylation is required for light chain association with the neck region to release myosin from its autoinhibited state (reviewed in Glotzer 2005). Upon activation, myosin tails dimerize to form filaments that can associate with actin and crosslink the cortex (reviewed in Cowan and Hyman 2007). In the absence of UNC-45, myosin can still bind actin but cannot contract suggesting that UNC-45 acts on myosin after myosin light chain activation and subsequent association with actin but prior to contraction.

The lack of cortical contraction is not only manifested as a polarity defect but also as a failure to complete cytokinesis. Our data suggests that UNC-45 is required for all stages of cytokinesis despite myosin still accumulating at the site of the presumptive furrow. Little is known about the recruitment of myosin to the site of the future cleavage furrow (for review see Matsumura 2005) and our results suggest that non-functional myosin is capable of being recruited to the furrow. In *Dictyostelium* the recruitment of myosin has been suggested to require a kinesin (Kif12) utilizing the central spindle (Lakshmikanth *et al.* 2004) providing a mechanism whereby non-functional myosin could be transported to the furrow. Alternatively, other myosin-associated proteins could recruit myosin to the cleavage furrow. For example, anillin has been shown to be required for myosin localization in fission yeast (Motegi *et al.* 2004) however it has not been shown to be required in *C. elegans* (Maddox *et al.* 2005).

The *C. elegans* germline provides a popular system to study the spatial regulation of the cell cycle. However a concurrent stage of meiosis, where nuclei get packaged into oocytes, has been largely unstudied. Our evidence suggests that the *C. elegans* germline provides an ideal system for studying the regulation and contractile mechanism of myosin. In the absence of UNC-45, and thus functional NMY-2, the gonad fails to establish membrane boundaries between proliferating nuclei. Therefore, a type II myosin (NMY-2) along with its partner chaperone (UNC-45) are required as the force generating system for all stages of germline cellularization. The earliest cellularization defect identified was at the L3 stage where membrane disorganization was apparent throughout the developing gonad. The L2 larvae did not show cellularization defects possibly reflecting a small contribution of myosin from the developing somatic gonad.

In germlines that fail to establish PGC boundaries there is a defect in nuclear progression and proliferation. We suggest that the aberrant nuclear progression is not indicative of loss of myosin function but rather due to a combination of the inability to respond to meiotic signals without an intact membrane and an overlap of microtubules that would normally be physically separated by the cellularization furrows. These meiotic spindles may overlap and invade adjacent PGCs resulting in incorrect segregation of DNA and the larger masses of DNA seen down the centre of the gonad.

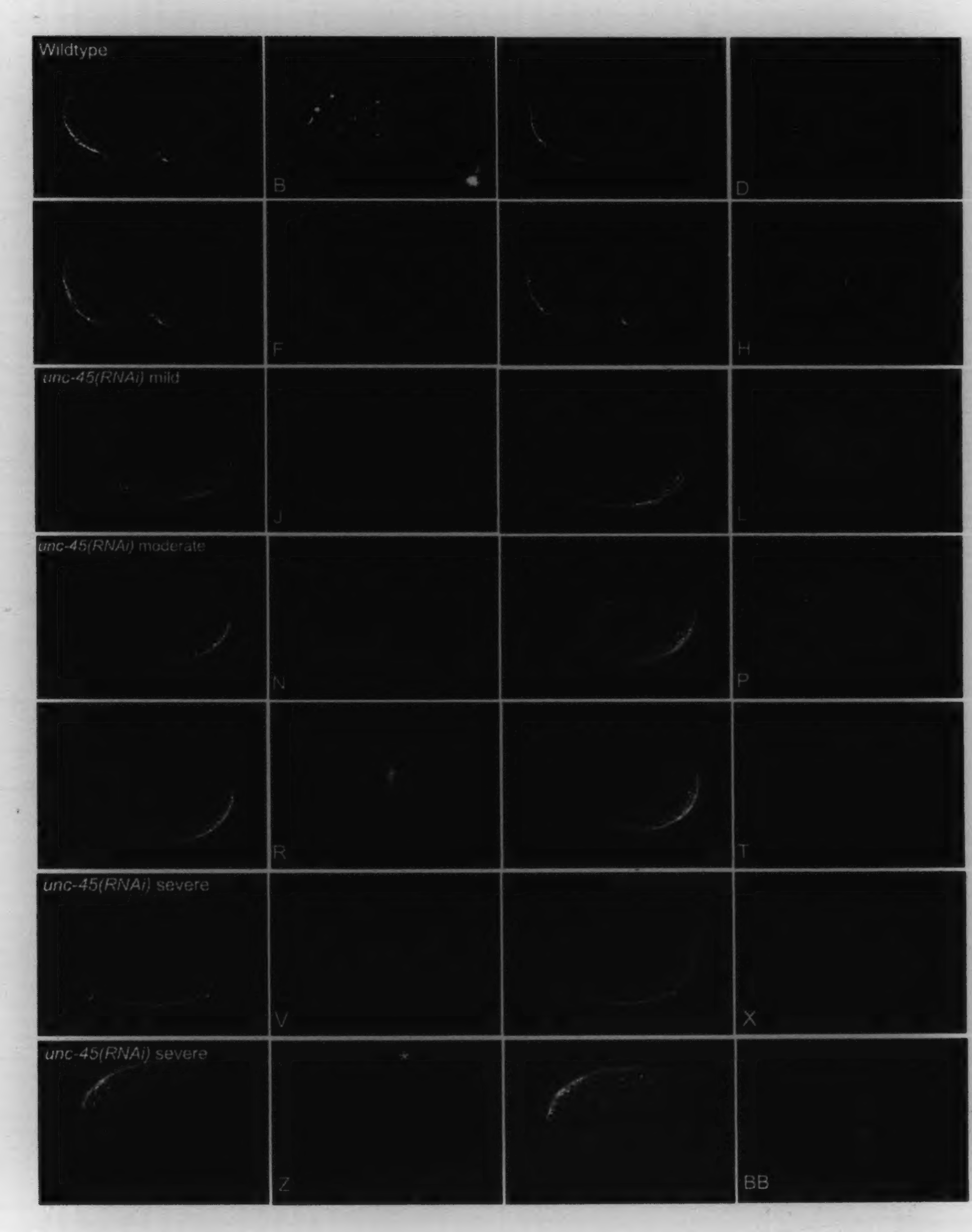
Germline cellularization has been mechanistically compared to cycle 13 embryonic cellularization in the *Drosophila* embryo (reviewed in Hubbard and Greenstein 2000). Many cytokinetic components are required in both cellularization events (reviewed in

Mazumdar and Mazumdar 2002; Piekny and Mains 2002; Shelton *et al.* 1999; Severson *et al.* 2002) and it was thought that these processes are highly analogous. Recent work in *Drosophila* has shown that the actomyosin cytoskeleton is only required during basal closure where the fully ingressed furrows come together to complete cellularization (Royou *et al.* 2004). Our results indicate that *C. elegans* non-muscle myosin II is required for all stages of membrane invagination suggesting that *Drosophila* cellularization, although sharing many characteristics, differs in the requirements for myosin.

Our identification of UNC-45 functions outside the body wall muscle has implications on the study of homologues in other organisms. It is clear that the UCS family members show considerable functional conservation as Rng3 in fission yeast interacts with a type II myosin during cytokinesis (Wong *et al.* 2000) and is required for the myosin II homologue to interact with actin (Lord and Pollard 2004). Two homologues of UNC-45 have been found in fish, mice and humans: a muscle specific homologue which appears to have a role similar to that in the worm body wall muscle and a second UNC-45 homologue is hypothesized to have a cytoskeletal function (Etheridge *et al.* 2002; Price *et al.* 2003; Wohlgemuth *et al.* 2007). Recently, the general cell isoform of UNC-45 was shown to be upregulated in multiple different cancer subtypes and GC-UNC-45 is required for ovarian cancer cell migration (Bazzaro *et al.* 2007). The function of nematode UNC-45 during cytokinesis and other contractile events in the embryo indicates that UNC-45 homologues in vertebrates may function during cell division and other microfilament related processes.

3.5 Figures

Figure 3.1. NMY-2::GFP localization in *unc-45(RNAi)* embryos. (A-H) DIC and GFP images of wildtype NMY-2::GFP embryos. (A) Wildtype pronuclear migration and pseudocleavage formation (asterisk). (B) Large contractile foci and retraction of NMY-2::GFP to anterior. Inset is image of largest foci magnified for size comparison to punctae in *unc-45(RNAi)* embryos. (C) Asymmetric pronuclear meeting. (D) Completion of anterior capping of NMY-2::GFP. (E) Asymmetric cytokinetic furrow. (F) NMY-2::GFP concentrates at furrow. (G) Completion of cytokinesis. (H) NMY-2::GFP contractile ring. (I-L) *unc-45(RNAi)* mild phenotype. (I) Pronuclear migration with failed ruffling. (J) NMY-2::GFP does not form an anterior cap. Inset shows myosin punctae that do not form large foci. (K) Near central pronuclear meeting. (L) NMY-2::GFP does not form distinct cortical domains. (M-T) *unc-45(RNAi)* moderate phenotype. (M) Pronuclear migration. (N) NMY-2::GFP clearing from site of sperm entry but no foci formation. (O) Pronuclear meeting. (P) Failed capping of NMY-2::GFP to anterior. (Q) Spindle elongation and attempted cytokinesis (arrow). (R) NMY-2::GFP concentrates at the furrow location. (S) Failed cytokinesis. Failed polar body extrusion (arrow). (T) Loss of NMY-2::GFP localization from the furrow. (U-X) *unc-45(RNAi)* severe phenotype. (U) Pronuclear migration. (V) Dispersal of NMY-2::GFP from site of sperm entry. (W) Pronuclear meeting. (X) NMY-2::GFP does not polarize to the anterior. (Y-BB) *unc-45(RNAi)* severe phenotype of an embryo with a lateral sperm entry position. (Y) Pronuclear meeting. (Z) Clearing of myosin away from sperm entry site. Asterisk denotes location of sperm entry. (AA) Spindle elongation. (BB) NMY-2::GFP concentrates at the failed cleavage furrow. Scale bar 25 µm.


Wildtype
B
D
F
H
unc-45(RNAi) mild
J
L
unc-45(RNAi) moderate
N
P
R
T
unc-45(RNAi) severe
V
X
unc-45(RNAi) severe
Z
BB

		Pronuclear Meeting (% Egg Length from Posterior)	Position of Cleavage Furrow (% Egg Length from Posterior)	Cortical Flow rate (µm/min)	Polar Body Extrusion (% of embryos)
Wildtype	Average	28% ± 2.8	42% ± 1.7	5.5	100%
RNAi	Average	47% ± 2.3	48% ± 1.8	0.00	7%
	n=	14	21	5 granules/3 embryos	14

Table 3.1. Defects in the microfilament cytoskeleton of *unc-45(RNAi)* depleted embryos. All classes of defects were combined in this analysis.

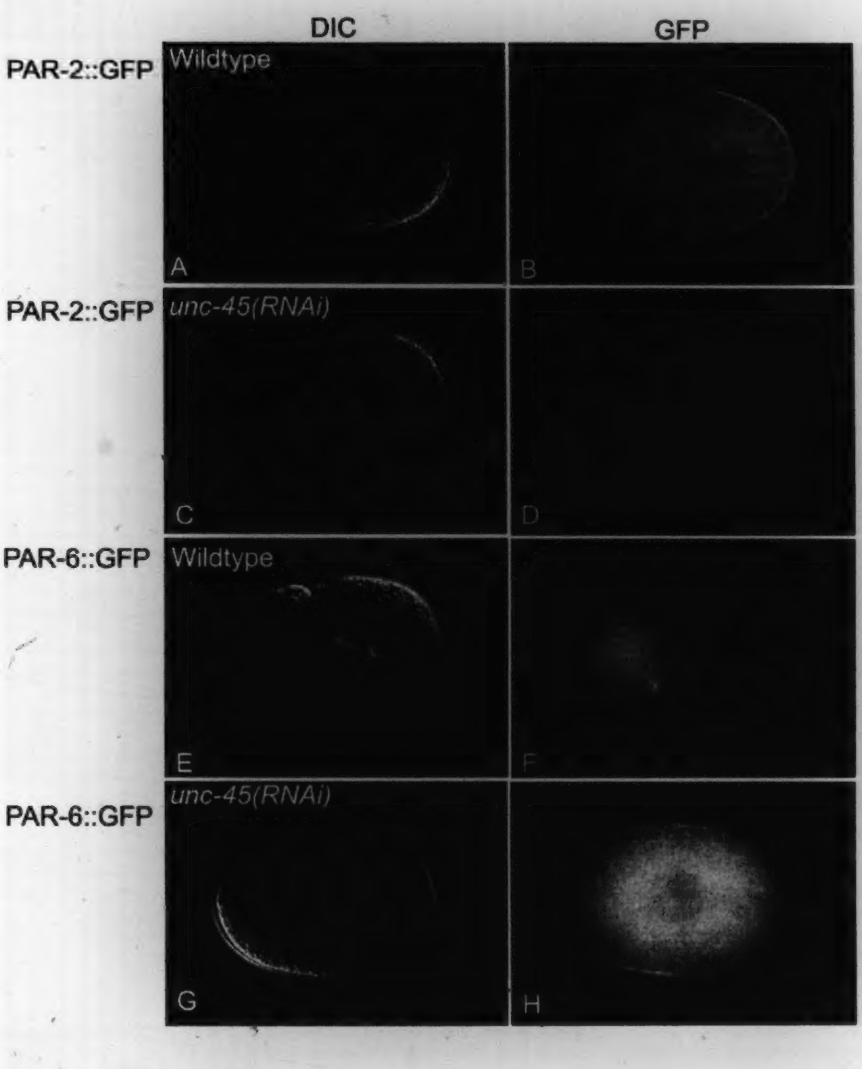
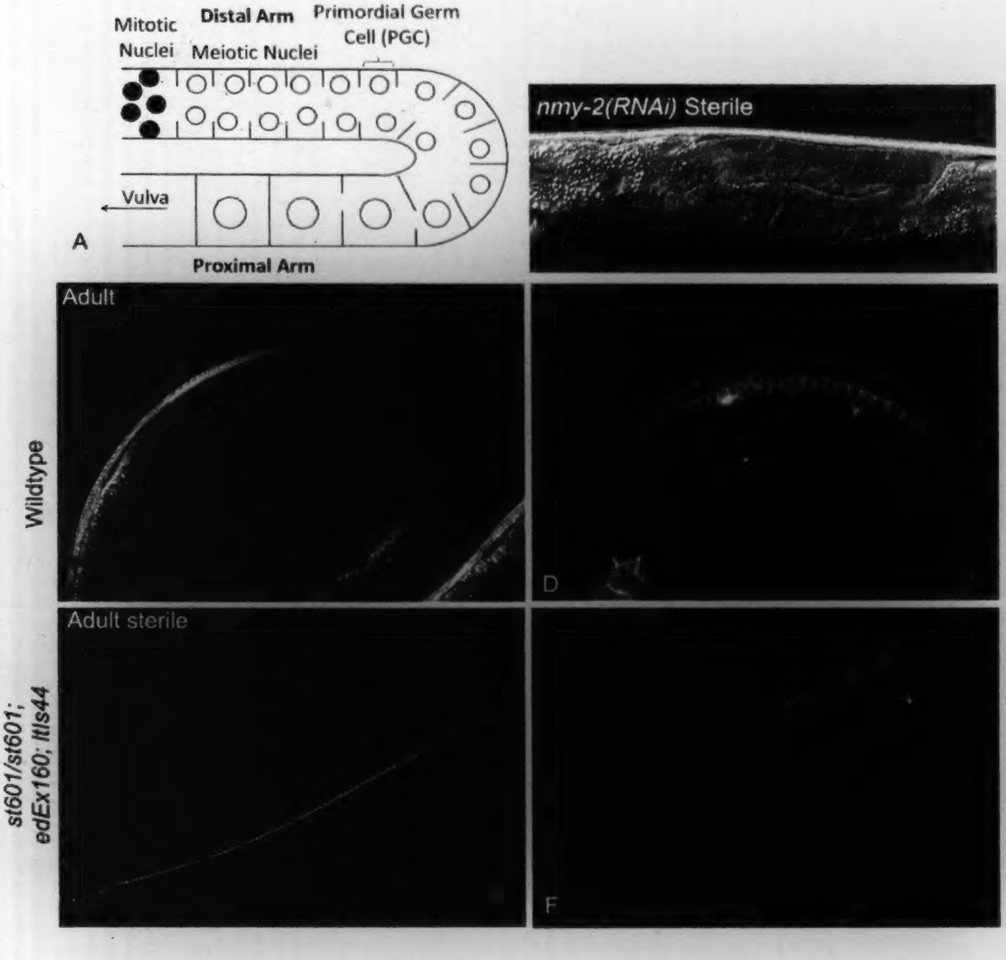

DIC
GFP
PAR-2::GFP
Wildtype
A
B
PAR-2::GFP
unc-45(RNAi)
C
D
PAR-6::GFP
Wildtype
E
F
PAR-6::GFP
unc-45(RNAi)
G
H

Figure 3.2. PAR protein localization in *unc-45(RNAi)* embryos. (A-B) Wildtype PAR-2::GFP images. (A) DIC of asymmetric pronuclear meeting. (B) Posterior cortical localization of PAR 2::GFP. (C) *unc-45(RNAi)* embryo exhibits central pronuclear meeting. (D) PAR-2::GFP fails to localize to the posterior cortex. (E-F) Wildtype PAR-6::GFP embryo shows anterior localization. (G) *unc-45(RNAi)* embryo exhibits failed polar body extrusion (arrowhead). (H) PAR-6::GFP localizes around the entire cortex in an *unc-45(RNAi)* embryo.

Figure 3.3. Membrane invagination in the germline requires UNC-45 and NMY-2. (A) Schematic of germline membrane organization. (B) *nmy-2(RNAi)* sterile adult hermaphrodite. (C) Wildtype adult hermaphrodite. (D) The membrane is organized and surrounds syncytial nuclei. (E) DIC of *st601/st601*; *ltIs44*; *edEx160* sterile gonad. (F) Membranes are disorganized and fail to invaginate in UNC-45 sterile adults. (G) Wildtype L4 hermaphrodite. (H) L4 cellularization furrows surround all nuclei. (I) Sterile L4 larvae. (J) Failed cellularization. (K) L3 wildtype hermaphrodite. (L) Cellularization furrows in the L3 gonad. (M) Sterile L3 hermaphrodite. (N) Failed germline cellularization in the early L3 hermaphrodite. (O) Wildtype L2 larvae. (P) L2 larvae membrane organization shows all visible nuclei with some degree of membrane invagination. (Q) *st601/st601; edEx160; ltIs44* L2 hermaphrodite. (R) Sterile L2 hermaphrodite encloses all nuclei by membranes. Asterisk denotes vulval or future vulval location.


Mitotic Nuclei
Distal Arm
Meiotic Nuclei
Primordial Germ Cell (PGC)
Vulva
A
Proximal Arm
nmy-2(RNAi) Sterile
Adult
Wildtype
D
Adult sterile
st601/st601; edEx160; ltIs44
F

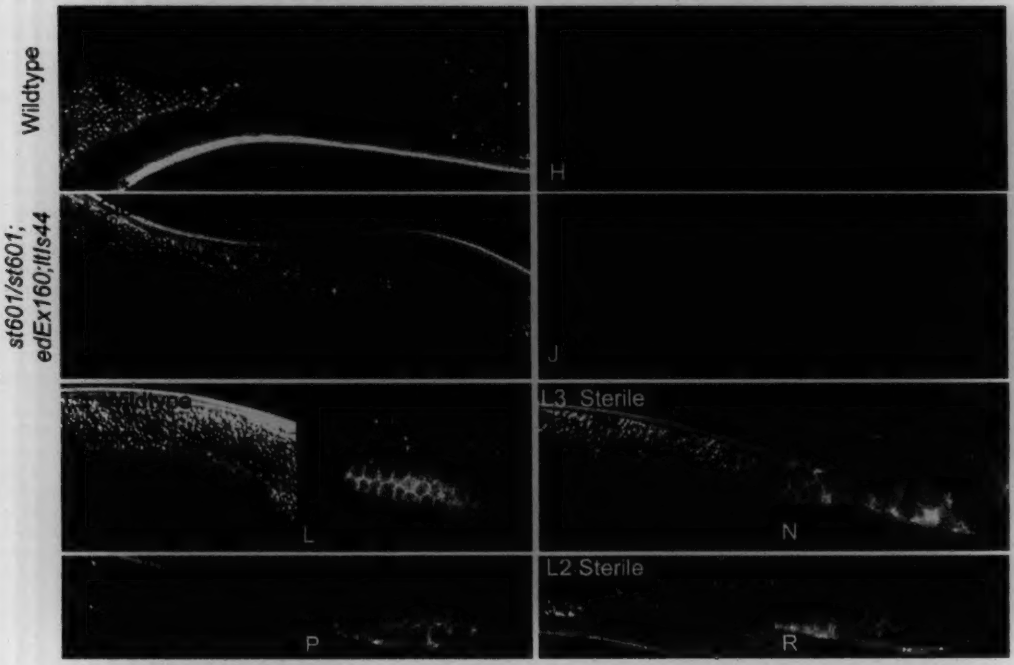
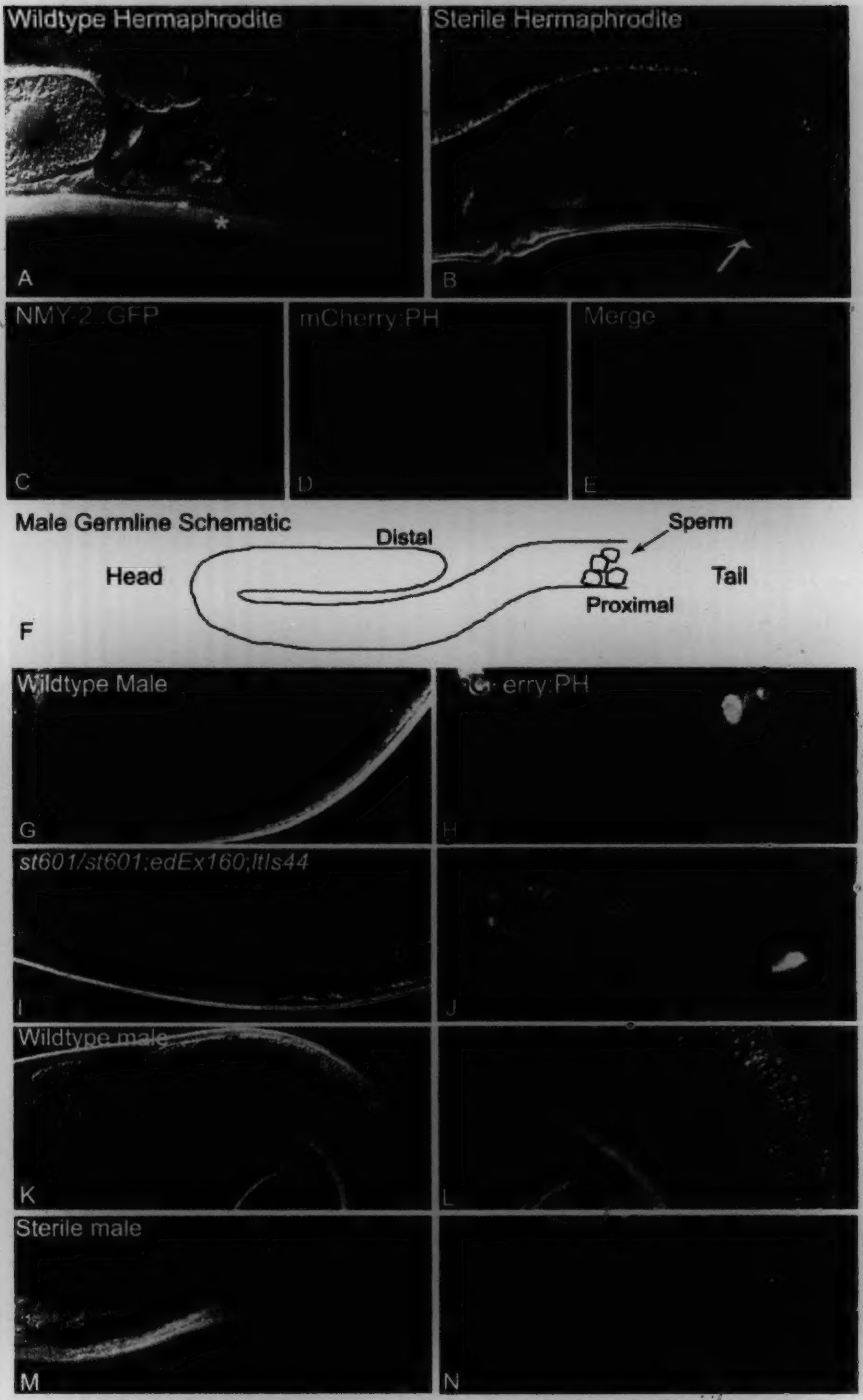


Figure 3.4. Spermatogenesis requires UNC-45 in both males and hermaphrodites. (A) Wildtype hermaphrodite has visible sperm in the spermatheca (asterisk). (B) Sterile hermaphrodite shows no sperm accumulation (arrow). (C) NMY-2::GFP in the distalmost gonad of an L4 hermaphrodite. (D) Membrane localization surrounding distal PGCs. (E) Merge of NMY-2::GFP and mCherry:PHPLCδPH. (F) Schematic of male germline. (G) Wildtype male. (H) Male gonad shows a membrane pattern that is similar to the hermaphrodite. (I) DIC of *unc-45* sterile male. (J) Sterile males fail to organize the germline membrane. (K) Wildtype sperm accumulation in the spermatheca. (L) Magnification showing individual sperm (arrow). (M) Failed sperm production in *unc-45* depleted males. (N) Magnification of the sterile male spermatheca. d denotes distal end of male gonad.


Wildtype Hermaphrodite
*
A
Sterile Hermaphrodite
B
NMY-2::GFP
C
mCherry:PH
D
Merge
E
Male Germline Schematic
Distal
Sperm
Head
Tail
Proximal
F
Wildtype Male
G
mCherry:PH
H
st601/st601;edEx160;ltIs44
I
J
Wildtype male
K
L
Sterile male
M
N

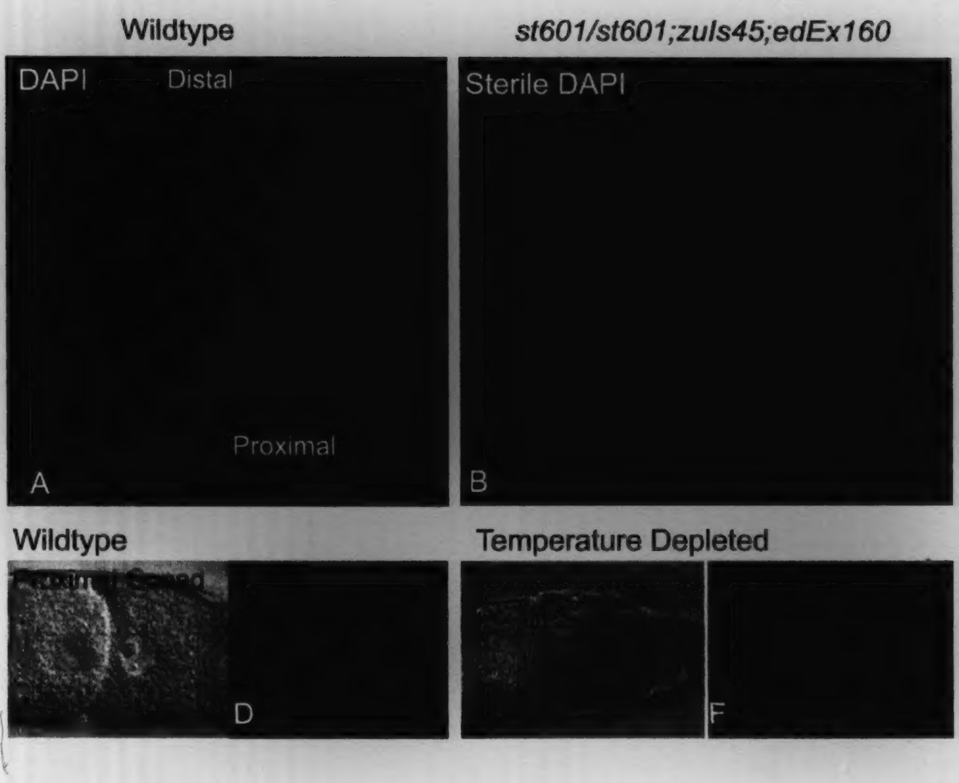

Wildtype
st601/st601;zuIs45;edEx160
DAPI
Distal
Proximal
A
Sterile DAPI
B
Wildtype
Proximal Gonad
D
Temperature Depleted
F

Figure 3.5. Nuclear proliferation in *unc-45* sterile hermaphrodites. (A) DAPI stain of wildtype gonad. (B) DAPI stain of dissected *st601/st601; zuIs45*; *edEx160* sterile hermaphrodite showing aggregation of nuclei. (C) DIC of wildtype proximal gonad showing two adjacent oocytes. (D) DAPI stain of wildtype nuclei. Distal is to the left in C-F. (E) Dinucleated oocyte in *unc-45* temperature depleted hermaphrodite in the proximal gonad. (F) Normal nuclear morphology in dinucleated oocyte.

NMY-2::GFP
Alexa-568 Phalloidin
Merge
Wildtype
st601/st601;zuIs45; edEx160
Sterile Magnification
A
B
C
D
E
F
G
H
I

Figure 3.6. Failure to organize the microfilament cytoskeleton in hermaphrodites lacking UNC-45. (A) Wildtype NMY-2::GFP dissected gonad. (B) Phalloidin staining reveals actin filaments organized into distinct boundaries for PGCs. (C) Merge of (A) and (B) showing co-localization. Scale bar = 25µm (D) Sterile hermaphrodite dissected gonad shows NMY-2::GFP does not form organized structures. (E) Actin filaments do not organize in *unc-45* sterile hermaphrodites. (F) Merge of NMY-2::GFP and Phalloidin staining shows co-localization of actin and myosin. (G-I) Magnification of *unc-45* sterile gonad showing regions of co-localization (asterisk). Scale bar = 25 µm.

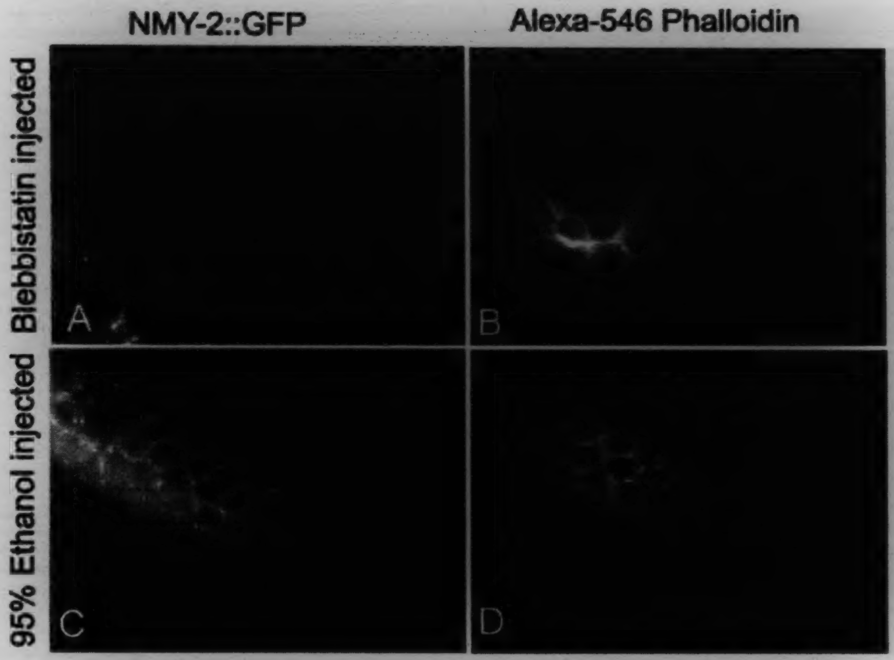

NMY-2::GFP
Alexa-546 Phalloidin
Blebbistatin injected
95% Ethanol injected
A
B
C
D

Figure 3.7. Blebbistatin treatment of NMY-2::GFP gonads. (A) Blebbistatin injected gonad results in myosin dissociating from the microfilament cortex. (B) Actin filaments exhibit normal arrangement in the distal gonad. (C) Control injections show NMY-2::GFP localizing to the microfilaments. (D) Actin filaments are unaffected by treatment.

3.6 References

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3.7 Appendix

3.7.1 RNA interference

RNA interference (RNAi) (Fire *et al.* 1998) is a routinely used technique for epigenetic silencing of genes in *C. elegans*. Various concentrations are used effectively with no reported dosage effects at excess concentrations. *C. elegans* and does not show dosage effects, toxic effects or lack of specificity (Rual *et al.* 2007) as with other RNA interference strategies in other systems, namely fish and mice. The RNAi experiments performed used concentrations of double-stranded RNA from 0.2 ng/µL to 0.6 ng/µL with no difference in severity of phenotype. Two different dsRNA constructs were used: DP#cU5 corresponds to exons 6 and 7 of the *unc-45* gene and is a 509 bp sequence with no predicted secondary targets in the *C. elegans* genome. DP#cU9 sequence corresponds to exon 5 and 6 and is a 644 bp sequence with no predicted secondary targets in the *C. elegans* genome. Predicted secondary targets were determined by a BLAST search on the *C. elegans* genomic database, WormBase. Purity and quality of the dsRNA was assayed using gel electrophoresis for the proper size of predicted dsRNA product and then concentration was determined using a NanoDrop that also detected any gross impurities in the *in vitro* transcription preparation. Therefore, *unc-45(RNAi)* is expected to be specific to that gene. Control RNAi injections were performed using 0.4 ng/µL *nmy-2* dsRNA and similar phenotype were observed as with previous published data (Guo and Kemphues 1996; Cuenca *et al.* 2002).

dsRNA was administered by microinjection into the body cavity or gonad of the worm. The location of administration does not matter in *C. elegans* as RNAi is effectively and efficiently transmitted through the body cavity (Fire *et al.* 1998; Grishok *et al.* 2000). Negative control injections were performed by injecting wildtype worms with distilled water.

3.7.2 Figures

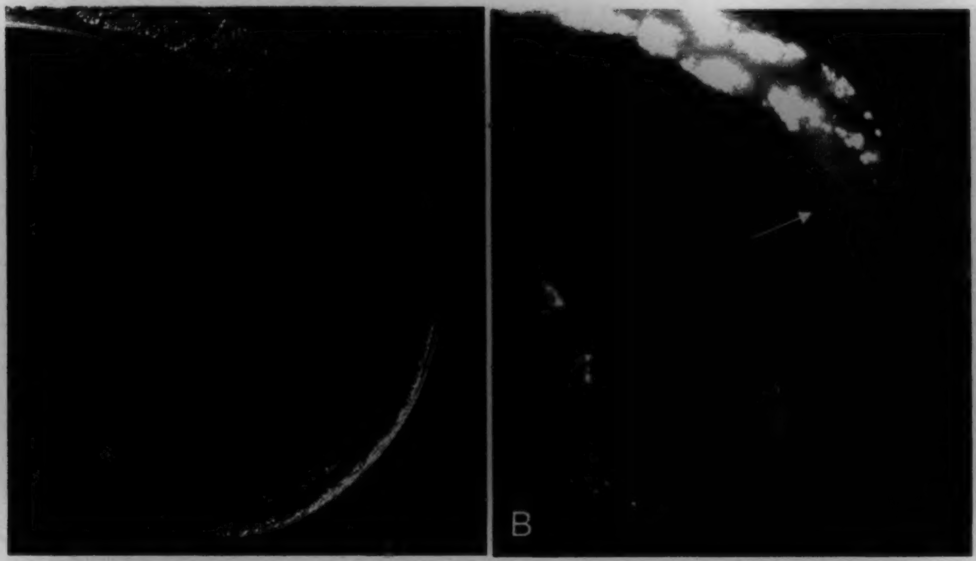

Figure 3.8. Localization of NMY-2::GFP in the male gonad. A) DIC microscopy of wildtype NMY-2::GFP male. B) NMY-2::GFP localization throughout male germline in a similar hexagonal pattern to hermaphrodites (arrow).

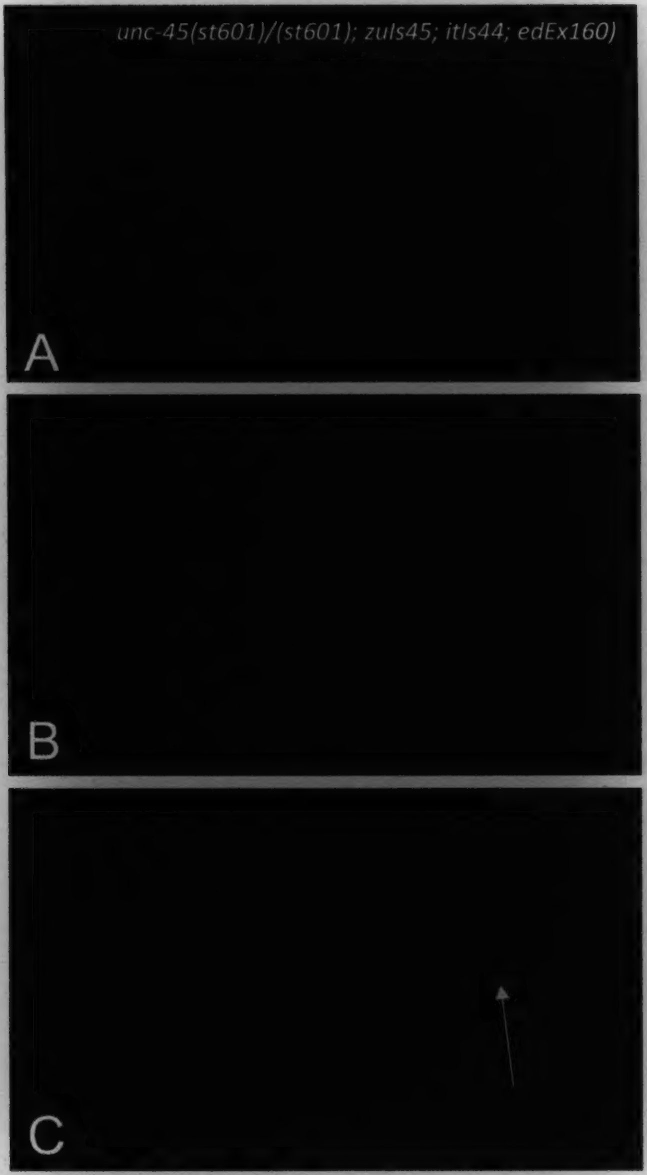
unc-45(st601)/(st601); zuIs45; itIs44; edEx160)
A
B
C

Figure 3.9. NMY-2::GFP remains membrane localized in *unc-45* sterile gonad. A) NMY-2::GFP pattern is disorganized when UNC-45 is depleted in the gonad. B) Membrane disorganization is disrupted (mCherry:PH) when UNC-45 is depleted. C) Myosin remains membrane localized despite lacking contractility in UNC-45 sterile animals (arrow).

4. Myosin Requirements and Regulation in the Germline of *C. elegans*

4.1 Introduction

Regulation of the contraction of myosin molecules is essential for maintaining coordinated muscle contraction, cell shape and cell movements. Type II myosins are the conventional two-headed myosin dimers involved in striated muscle contraction as well as non-muscle functions. These myosin molecules consist of two heavy chains, two essential myosin light chains and two regulatory light chains (rMLC) that allow myosin contraction. The attachment of myosin to actin requires phosphorylation of the regulatory myosin light chains by several possible kinases to release myosin from its autoinhibited state (Glotzer 2005). Two such kinases are the Rho-dependent kinase (ROCK) and myosin light chain kinase (MLCK) that phosphorylate rMLC on the Ser19 residue to release the inhibitory folding of the myosin tail and allow actin binding (Matsumura 2005). *let-502* is the only ROCK homolog in *C. elegans* that is expressed in the germline but does not show an sterile phenotype consistent with an early defect in primordial germ cell formation when knocked down either genetically or by RNAi (Piekny and Mains 2002). Therefore there is either redundancy in the pathway or two separate regulatory molecules.

In *C. elegans* two type II non-muscle myosin molecules are expressed, *nmy-1* and *nmy-2*, where *nmy-2* has received considerable attention for its role in polarizing the early embryo (Cuenca *et al.* 2003; Munro *et al.* 2004). NMY-1 has been shown to be required for elongation of the embryo but earlier roles during embryogenesis have not been found (Piekny *et al.* 2003). Despite having two non-muscle myosins, *C. elegans* only contains one regulatory light chain for non-muscle myosin, MLC-4 that is required for polarity and cytokinesis in the early embryo and has additional roles during elongation (Shelton *et al.* 1999; Piekny *et al.* 2003). Both NMY-2 and MLC-4 are required during embryonic cytokinesis for the formation of the contractile ring (Guo and Kemphues 1996; Shelton *et al.* 1999); additionally, both NMY-2 and MLC-4 exhibit sterility in RNAi screens but the basis for this sterility has not been fully examined (Kamath and Ahringer 2003).

Cellularization of the hermaphrodite gonad requires a modified form of cytokinesis to develop both sperm and oocytes. Cellularization begins at the distal end of the gonad but the nuclei remain in a syncytium and connected to the rachus. As the primordial germ cells enter the proximal gonad they increase in size due to cytoplasmic flows (Wolke *et al.* 2007) and are formed into oocytes from the syncytium by closing the rachus windows (Hubbard and Greenstein 2000). Studies have shown that myosin is required for cellularization in the *Drosophila* embryo, but only for the final stages, where the membrane is constricted inwards to complete the encircling of the cell instead of the formation of the lateral membranes between nuclei (Royou *et al.* 2004).

We have identified two distinct phases of cellularization in the germline where lateral growth and apical closure are two distinct regulatory events. Our results indicate that myosin is required for both lateral growth and apical closure and that these phases must be distinctly regulated at the level of myosin contraction. NMY-2 is required for all stages of germline cellularization whereas the regulatory light chain (MLC-4) is only required for apical closure. This suggests a second myosin light chain must be acting in the PGCs to establish lateral membranes and two separate genetic pathways must be controlling these two phases. Additionally two independent myosin regulators must be involved, likely to control these phases of membrane addition and growth. Therefore we show that a second regulatory light chain must be required but there is no clear candidate for a second non-muscle rMLC. Our work represents a preliminary characterization of the mechanics of cellularization and requirements for regulation.

4.2 Materials and Methods

4.2.1 Nematode cultures and strains

C. elegans strain N2 (wild type) was obtained from the stock collection of the MRC Laboratory of Molecular Biology, Cambridge, UK and maintained as previously described (Wood 1988). The NMY-2::GFP (*zuIs45*) strain was kindly provided by E. Munro (Munro *et al.* 2004). The maternal MLC-4::GFP strain was kindly provided by A. Hyman (Max Planck Institute, Dresden, Germany). The *mlc-4(or253)* deletion strain (EU618) and somatic transgenic array (EU573) were kindly provided by B. Bowerman (University of Oregon, Eugene, Oregon, USA) (Shelton *et al.* 1999). Sterile animals are homozygous for the lethal allele and somatically rescued for *mlc-4* by a transgene, but not rescued in the germline because transgenic arrays are often silenced in the germline (Kelly and Fire 1998). To visualize the germline membrane an mCherry:pleckstrin homology domain fusion was used (Kachur *et al.* 2007).

4.2.2 RNA interference

RNAi (Fire *et al.* 1998) was performed by microinjection of dsRNA into L4 or young adult hermaphrodite gonads. *nmy-2(RNAi)* was prepared from a 1182 bp fragment of the gene. For visualization of sterile gonads in vivo, adult worms were anaesthetized in M9 with 0.1% tricaine and 0.01% levamisole and mounted on 3% agarose pads.

4.2.3 Fluorescence Microscopy

Staining of actin was performed as described below on dissected hermaphrodites in 1x sperm salts (50 mM PIPES, 25 mM [illegible]Cl, 1 mM $MgSO_4$, 45 mM NaCl, 2 mM $CaCl_2$) on poly-L-lysine slides (Erie Scientific) then fixed in 3% paraformaldehyde. Gonads were permeabilized by freeze cracking, immersed in 95% ethanol for 1 min and washed in 0.1% PBS-Tween. Microfilaments were visualized by adding 0.02 units Alexa-546 labeled phalloidin to the specimens (Molecular Probes). NMY-2::GFP signal was maintained after the fixation procedure and GFP was visualized without antibody addition. Images were taken using Retiga EXi camera mounted on a Zeiss Axioscope 2 fitted with a 63 x 1.4 N.A. lens and a mercury lamp.

4.3 Results

4.3.1 NMY-2 is required for all phases of germline cellularization

NMY-2 depletion in the germline causes complete sterility because membranes do not undergo lateral growth or closure to surround the syncytial nuclei to generate primordial germ cells. Normally the wildtype gonad consists of a distal arm that contains the mitotic and meiotic nuclei and as nuclei proceed into the proximal gonad they are cellularized into large oocytes (Figure 4.1A). Membrane barriers form between each nucleus that is arranged near the surface of the gonad (Figure 4.1B) with small windows that connect the PGCs to the central rachus. *nmy-2(RNAi)* treated worms are completely sterile with no oocytes in the proximal gonad (Figure 4.1C). In *nmy-2(RNAi)* worms there is no evidence of any membrane invagination into the centre of the rachus suggesting that myosin is required for the lateral growth phase. NMY-2 is also required for the closure phase as partial depletion of *nmy-2* results in failed oogenesis (Figure 4.1E) despite proper lateral invagination and formation of a rachus (Figure 4.1F arrow). In the absence of functional myosin the actin cytoskeleton is grossly disorganized (Figure 4.1H) with some filaments protruding into the centre of the gonad but not in a consistent or organized pattern.

4.3.2 MLC-4 is not required for the lateral growth phase of cellularization

MLC-4 is thought to be the sole regulatory light chain for non-muscle myosin in the worm and depletion results in reported sterility and embryonic defects such as failed cytokinesis and polarity establishment (Shelton *et al.* 1999). It was expected that the components that regulate myosin would exhibit a similar severity of defect as the *nmy-2(RNAi)* depletion. NMY-2::GFP normally localizes to both the lateral membranes and rachus windows (Figure 4.2B) in agreement with NMY-2 being required for both phases of cellularization.

Worms depleted for *mlc-4* in the germline but rescued in the soma by a transgenic array exhibit sterility (Shelton *et al.* 1999). However, the degree of the cellularization defect is not as severe as an *nmy-2* depleted germline; there is considerable and organized lateral membrane ingression generating primordial germ cells. Oocytes are visible in the proximal gonad suggesting that cellularization can occur but some are polynucleate (Figure 4.2C arrow). NMY-2::GFP still localizes to the windows of the rachus but lateral membrane localization is substantially reduced (Figure 4.2D and F). General nuclear organization in the distal gonad appears normal without the large disruption in nuclear morphology that occurs in germlines lacking *nmy-2* (Figure 4.2E). Some primordial germ cells in the distal gonad are polynucleated (Figure 4.2E arrow) possibly representing the occasional failure in lateral membrane formation. However, MLC-4 is not required to the same degree as NMY-2 for cellularization of the germline despite MLC-4 being the only identified regulatory light chain for non-muscle myosin.

4.3.3 Localization of MLC-4 in the germline

When MLC-4::GFP is expressed from the *pie-1* promoter in the germline, it localizes to the rachus throughout a normal and fertile gonad with pronounced localization at the rachus windows (Figure 4.3B). MLC-4::GFP does not localize to the lateral membranes in the distal gonad but does accumulate on the lateral membranes at the distal to proximal turn of the gonad (Figure 4.3B arrow). Therefore MLC-4::GFP localization does not mirror NMY-2::GFP localization because NMY-2::GFP is present on all cellularization membranes throughout the proximal and distal gonad.

4.3.4 Myosin-actin binding in the absence of MLC-4

In order to determine whether myosin can bind actin in the absence of the regulatory myosin light chain (MLC-4) we performed a phalloidin stain to determine if myosin has reduced co-localization with actin in the absence of rMLC. Normally actin and myosin strongly co-localize as shown by phalloidin and NMY-2::GFP patterns (Figure 4.4A-C). In *mlc-4* sterile gonads actin organization was disrupted and accumulated in the rachus without any consistent pattern (Figure 4.4D). Similarily, NMY-2::GFP pattern was disrupted with formation of a rachus window (Figure 4.4E). However, in MLC-4 sterile gonads myosin co-localized with actin (Figure 4.4F) suggesting that myosin can still bind actin in the absence of this MLC-4 regulatory light chain. A caveat to this conclusion is that the or253 allele of *mlc-4* is a null allele, this is predicted because it is a truncation allele that deletes the majority of the gene (Shelton *et al.* 1999).

4.3.5 Preliminary characterization of regulators required for cellularization

The activation of rMLC by phosphorylation has multiple potential inputs including small GTPases such as Rho. *C. elegans* only contains one Rho homolog (RHO-1) that is regulated by multiple GEFs and GAPs. ECT-2 is a RhoGEF that is an activator of myosin light chain phosphorylation (Figure 1.15). ECT-2 is not known to activate myosin through any other phosphorylation cascade besides Rho (Morita *et al.* 2005). *ect-2* mutants are partially sterile with incompletely cellularized oocytes (Figure 4.5B and C) despite its potential effector *let-502* having a less severe phenotype in a variety of mutant alleles (Wissman *et al.* 1999; Piekny and Mains 2002). More severely affected alleles of *let-502 (h392, h509* and *h732)* also result in sterility; however, some dead eggs are occasionally produced suggesting that this phenotype is a result of a defect in a late stage of oocyte development (Wissman *et al.* 1999). This suggests that ECT-2 regulates myosin through two pathways – LET-502 and a second activation pathway. CDC-42 is a Rho-like small GTPase that participates in cytokinesis in the embryo (Motegi and Sugimoto 2006) but *cdc-42* mutants do not show a defect in cellularization but rather exhibit a spermatogenesis defect suggesting that CDC-42 may participate in both spermatogenesis and possibly oocyte maturation (Schmutz *et al.* 2007)(Figure 4.5D). It is predicted that ECT-2 acts in both the lateral growth phase and apical closure phase of cellularization but LET-502 is only required during apical closure. CDC-42, which acts with ECT-2 in the embryo (Motegi and Sugimoto 2006; Schonegg and Hyman 2006) does not participate in myosin regulation during cellularization. Therefore a RHO-1-associated kinase is likely involved in the cellularization process along with other kinases that can activate rMLC.

4.4 Discussion

The mechanism of cellularization in *C. elegans* requires two distinct phases of myosin contraction, the first to establish the lateral membrane walls that form the primordial germ cells and secondly, the closing of the rachus windows to complete cellularization. In order to establish two unique phases of myosin contraction there must be separate regulatory events to control myosin contractility. There is a lateral growth phase that occurs early in the distal germline that does not require myosin regulatory light chain MLC-4 and an apical closure phase in the proximal germline that requires MLC-4. Despite MLC-4 being the only predicted regulatory light chain in *C. elegans* (Shelton *et al.* 1999), both phases require NMY-2 and its chaperone UNC-45 (Kachur *et al.* 2007). We have shown that NMY-2 is required for all stages of germline cellularization in *C. elegans.* In the absence of NMY-2 the PGC membranes fail to invaginate into the rachus, the actin cytoskeleton is disorganized and both sperm and oocytes do not form. However, the predicted sole regulatory light chain MLC-4 (Shelton *et al.* 1999) is only required for apical closure and localizes predominantly to the rachus windows and not along the lateral membranes in the distal germline. However, in the absence of MLC-4 NMY-2 can still bind actin. This suggests that an additional regulatory light chain for non-muscle myosin is required for lateral growth. In the proximal germline the membranes grow into the rachus of the gonad and stall until apical closure at the proximal to distal transition. Finally, if myosin is required for both phases, but these membrane growth phases are temporally and spatially distinct, then different regulatory events must control these processes at the level of myosin. We show that

ECT-2 but not CDC-42 functions upstream to regulate cellularization but additional factors must be involved.

Candidate genes for regulators of rMLC phosphorylation are myosin light chain kinases (MLCK), citron kinases, Rho-associated kinases and Pak p21 associated kinases (Matsumura 2005). *C. elegans* contains several homologs within all of these classes but none have been identified as involved in cellularization by various RNAi screens (Table 4.1) (Sonnichsen *et al.* 2005; Lehner *et al.* 2006; Kamath and Ahringer 2003). Therefore there are likely many redundancies in controlling myosin light chain phosphorylation. Many of the identified kinases that could target rMLC do not show mRNA expression in the germline (Dr. Y. Kohara, online database at http://nematode.lab.nig.ac.jp/ dbest/srchbyclone.html) (Table 4.1) but some promising candidates are *unc-43* and *unc-22* that show sterility in RNAi screens (Corrigan *et al.* 2005; Lehner *et al.* 2006). Both of these genes are myosin light chain kinase homologs (MLCK) that may cooperate with *let-502*, a Rho-associated kinase, in a redundant fashion to control myosin contractility. But *unc-43* is required for oocyte maturation and not cellularization likely eliminating this gene from possible cellularization regulators (Corrigan *et al.* 2005). As well, *unc-22(RNAi)* experiments that resulted in sterility had a secondary target of *mom-2* which may be required for Wnt signaling in the gonad (Lehner *et. al.* 2006). Thus, we are left with no clear candidates for MLC homologs or regulators.

Although the kinases that control MLC-4 phosphorylation have not been identified one of the upstream signaling pathways can be determined to be a RHO-1 dependent pathway. Upstream regulators of RHO-1, proteins such as ECT-2 and RGA3/4 have defects in positioning of the cellularization furrows (Schmutz *et al.* 2007), which may account for the sterility observed in ECT-2 knockdowns. ECT-2 is required for cortical contractility in the embryo while loss of RGA-3/4 increases contractility (Schonegg and Hyman 2007). *ect-2* depleted gonads decreased myosin based contractility resulting in polynucleated oocytes and PGCs (Figure 4.5). Preliminary characterization of an *rga-3/4* (RNAi) gonad exhibits failed lateral growth of the membranes with only very small PGCs being formed (Schmutz *et al.* 2007). These gonads appear to properly organize actin around the rachus windows suggesting that loss of RGA-3/4 causes hypercontractility and premature attempts at cellularization, some of which fail to enclose a nucleus thus giving rise to the fallen-nuclei phenotype. Whereas *ect-2* mutants have decreased contractility and decreased cellularization.

Calmodulin dependent protein kinases are also capable of phosphorylating regulatory myosin light chains but do not function in the early embryo to control cytokinesis (Batchelder *et al.* 2007). *cmd-1* is expressed throughout the gonad (Batchelder *et al.* 2007) and RNAi experiments show sterility, therefore calmodulin and its downstream kinases may be required for cellularization. Regardless of the evidence suggesting a potential role for calmodulin-related proteins in *C. elegans* germline, there is no obvious downstream effector of calmodulin, namely a MLCK. Therefore calmodulin-related family members may not function through an MLCK and may not function directly with myosin.

We have characterized two distinct phases of myosin regulation during cellularization such that lateral growth between meiotic nuclei can occur throughout the distal germline but apical closure to complete cellularization of germ cells occurs only distally in the germline. Our data also suggest that another regulatory myosin light chain is likely involved in the lateral growth phase that uses NMY-2 as the myosin involved. Our results may reflect an incomplete depletion of MLC-4 in the germline and RNAi experiments should be conducted to confirm this phenotype. An incomplete depletion of MLC-4 is unlikely, however, because the *or253* allele represents a 1 kbp deletion of the 1.13 kbp *mlc-4* gene (Shelton *et al.* 1999). There are no obvious other regulatory myosin light chain homologs in *C. elegans* except *mlc-1* and *mlc-2* neither of which are required or expressed in the germline.

Cellularization provides a very interesting and easy organ to study non-muscle myosin regulation and is the first process found in *C. elegans* to require different myosin regulation in a spatiotemporal fashion. Our research implicates a second regulatory myosin light chain in addition to MLC-4, but, there are no obvious homologs except for the muscle and pharynx rMLC proteins MLC-1 and MLC-2 neither of which is maternally expressed. Therefore our findings may reflect a small contribution of MLC-4 from the somatic transgene to the germline or perhaps additional factors that provide the same function may be found by future genetic screens.

4.5 Figures

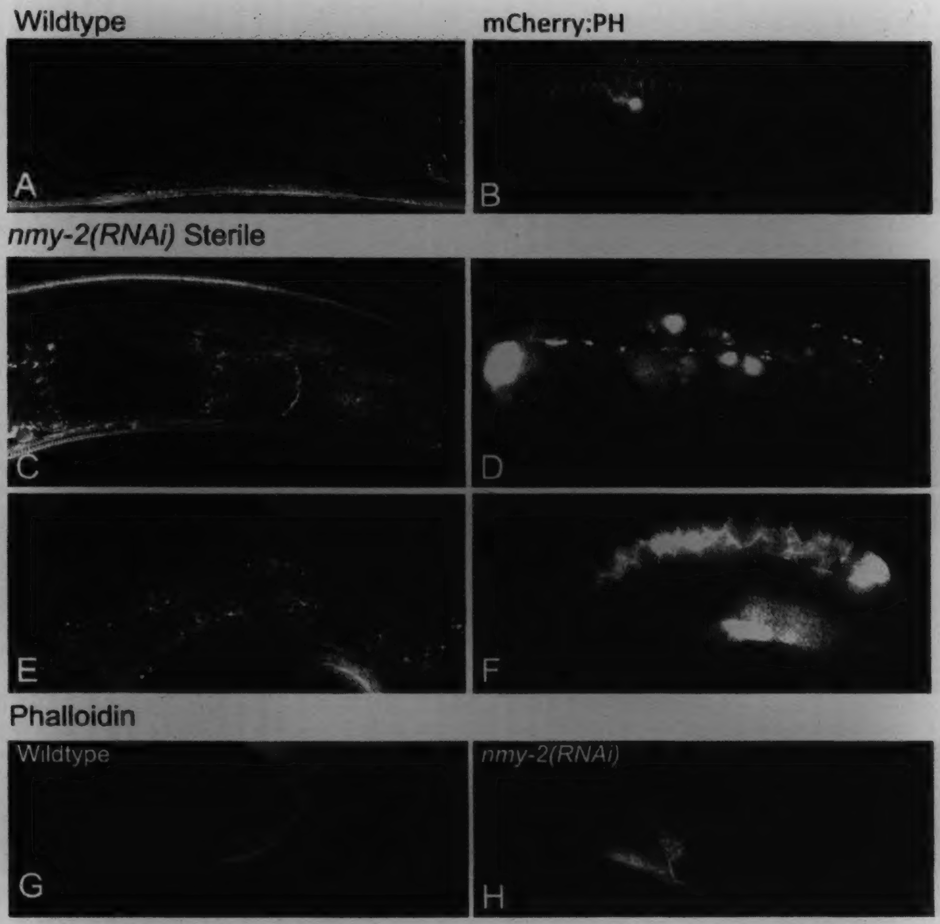
Wildtype
mCherry:PH
A
B
nmy-2(RNAi) Sterile
C
D
E
F
Phalloidin
Wildtype
nmy-2(RNAi)
G
H

Figure 4.1. *nmy-2(RNAi)* sterility. A) Wildtype gonad DIC. B) mCherry::PH localization to the cellularization membranes. C) *nmy-2(RNAi)* sterility with failed cellularization and no formation of oocytes in the proximal gonad. D) mCherry:PH localization in *nmy-2(RNAi)* sterile hermaphrodites. E) *nmy-2(RNAi)* partial sterility. F) mCherry:PH partial membrane invagination with the formation of a rachus (arrow). G) Wildtype phalloidin stain of distal gonad. H) Organization of microfilaments in *nmy-2(RNAi)* hermaphrodite gonad.

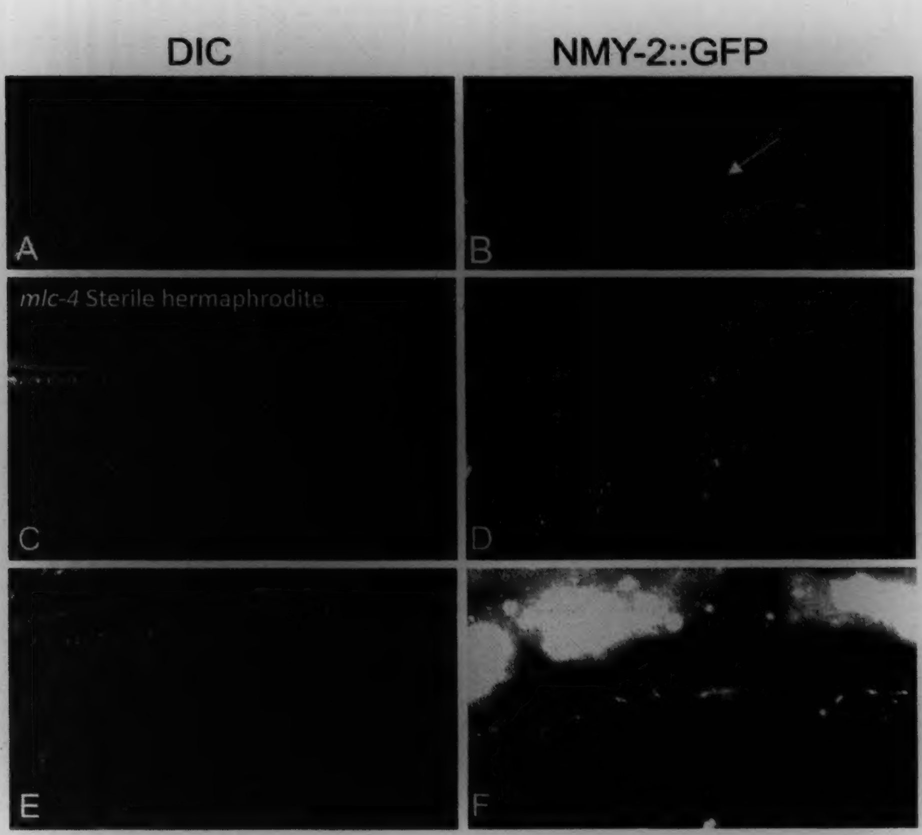

Figure 4.2. Distribution of NMY-2::GFP in *mlc-4* sterile hermaphrodites. A) Wildtype gonad. B) NMY-2::GFP localization in wildtype to PGCs but absent from rachus (arrow). C) *mlc-4* sterile hermaphrodite. D) NMY-2::GFP distribution in *mlc-4* sterile hermaphrodite. E) Magnification of distal gonad in *mlc-4* sterile hermaphrodite showing polynucleate PGCs (arrow). F) Localization of myosin to rachus windows and weak localization to lateral membranes in *mlc-4* sterile hermaphrodites.

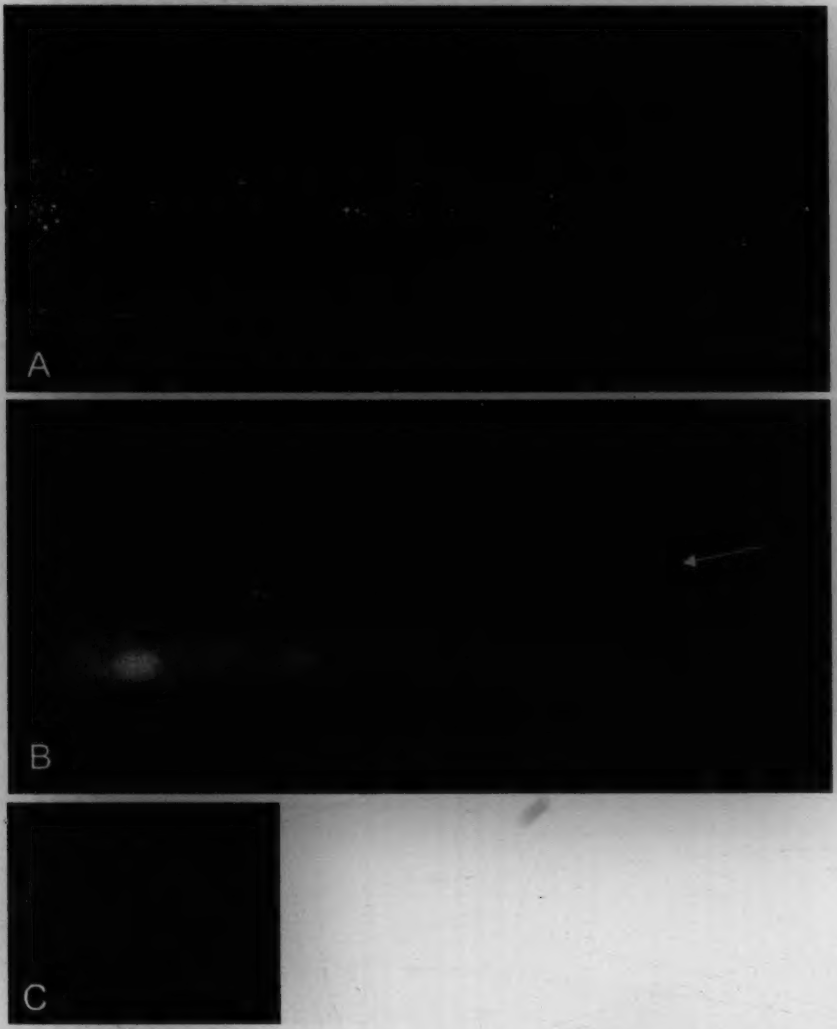

Figure 4.3. Localization of MLC-4::GFP in wildtype. A) DIC of MLC-4::GFP hermaphrodite. B) Distribution of MLC-4::GFP in wildtype with the accumulation of MLC-4::GFP at the lateral membranes at the turn of the gonad (arrow). C) Magnification of a single oocyte with both lateral and apical MLC-4::GFP localization.

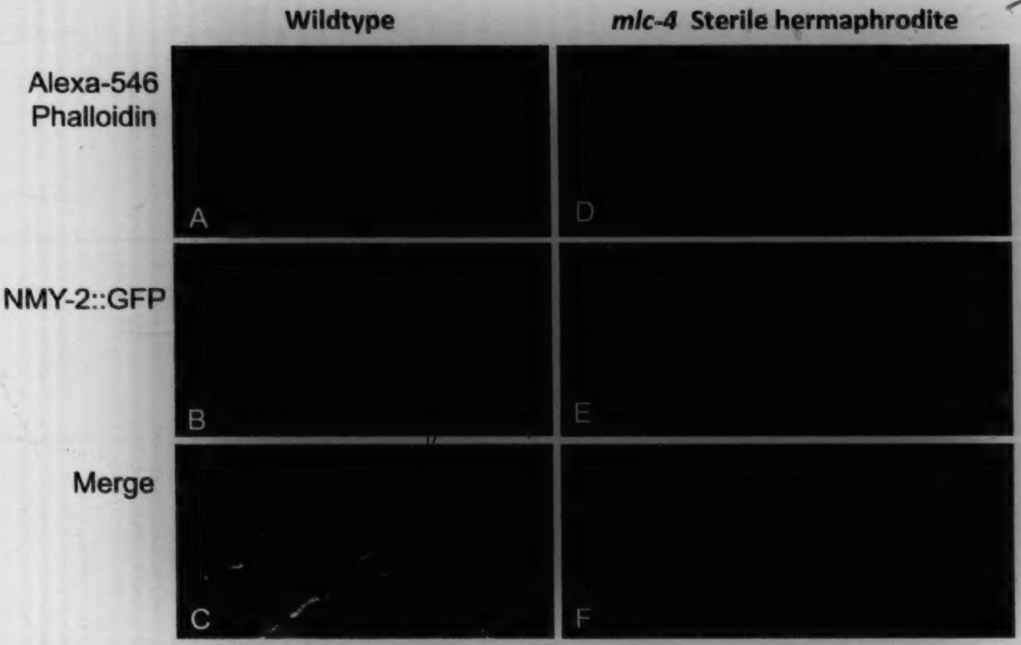
Wildtype
mlc-4 Sterile hermaphrodite
Alexa-546 Phalloidin
NMY-2::GFP
Merge
A
B
C
D
E
F

Figure 4.4. Distribution of myosin and actin in *mlc-4* sterility. A) Wildtype microfilament distribution. B) Wildtype NMY-2::GFP distribution. C) Co-localization of actin and myosin in wildtype distal gonad. D) Distribution of microfilaments in *mlc-4* sterile animals. E) Distribution of NMY-2::GFP in *mlc-4* sterile animals. F) Co-localization of myosin and actin in the absence of MLC-4.

Wildtype
Adult *ect-2* mutant
Late L4 *ect-2* mutant
cdc-42 mutant

Figure 4.5. Myosin light chain regulators. A) Wildtype cellularization. B) *ect-2* homozygous adult has polynucleated oocytes (arrow) and decreased cellularization. C) *ect-2* L4 larvae shows decreased cellularization throughout distal gonad. D) *cdc-42* mutant adult has successfully completed cellularization but fails to fertilize and mature oocytes.

	Gene name	Sterile RNAi phenotype?	Germline Expression?	Reference
MLCK candidates	ttn-1	No	mRNA expression in vulva.	Sonnichsen *et al.* 2005; Kamath and Ahringer 2003.
	unc-22	Sterile	No mRNA detectable in gonad.	Lehner *et al.* 2006.*
	cmk-1	No	No mRNA detectable in gonad.	Sonnichsen *et al.* 2005
	unc-43	Sterile	Expressed in oocytes and localizes with F-actin.	Corrigan *et al.* 2005.
	dapk-1	No	No mRNA detectable in gonad.	Sonnichsen *et al.* 2005; Fraser *et al.* 2000.
	ZC373.4	No	mRNA expressed strongly in extreme proximal gonad and sperm.	Sonnichsen *et al.* 2005; Maeda *et al.* 2001.
	unc-89	No	mRNA expressed throughout gonad especially in distal gonad.	Kamath and Ahringer 2003; Sonnichsen *et al.* 2005
Citron kinase candidates	F59A6.5	No	mRNA strongly expressed in gonad.	Kamath and Ahringer 2003; Sonnichsen *et al.* 2005
	tag-59	No	mRNA strongly expressed in gonad.	Kamath and Ahringer 2003; Sonnichsen *et al.* 2005; Maeda *et al.* 2001.
	T08G5.5	No	mRNA expressed in gonad.	Kamath and Ahringer 2003; Sonnichsen *et al.* 2005
	W02B8.2	No	mRNA weakly expressed in gonad.	Kamath and Ahringer 2003; Sonnichsen *et al.* 2005
	gck-2	No	mRNA is ubiquitous including in gonad.	Kamath and Ahringer 2003; Sonnichsen *et al.* 2005
	mig-15	No	Weak mRNA expression in proximal gonad.	Kamath and Ahringer 2003; Sonnichsen *et al.* 2005

* RNAi experiments were conducted in the RNAi sensitized *rrf-3* background

Table 4.1. Candidate kinases for regulation of rMLC. All potential Myosin Light Chain Kinases (MLCK) or Citron Kinase homologs were obtained and sterility in RNAi experiments was determined. Adapted from Batchelder *et al.* 2007.

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5. General Discussion and Conclusions

UNC-45 plays a critical role in promoting myosin function in the body wall muscle of *C. elegans* by acting as a co-chaperone with Hsp90 to fold the contractile myosin head (Barral *et al.* 2002). However, *unc-45* mutants showed phenotypes that were more severe than mutations in the muscle myosin MHC B (Venolia and Waterston 1990), therefore additional roles for maternally provided UNC-45 were proposed.

Characterization of the maternal contribution of UNC-45, performed by genetic knockdowns of *unc-45* in a heteroallelic temperature depleted background, has revealed important roles in the embryo during cytokinesis that were (Kachur *et al.* 2004; discussed in section 2). We determined that UNC-45 interacts with a Type II non-muscle myosin, NMY-2 which is also required for cytokinesis (Guo and Kemphues 1996). Therefore it was hypothesized that maternally provided UNC-45 was required to act on NMY-2 in a similar fashion to its role in the body wall muscle with MHC B. Our results provided insight into the mechanism of UNC-45 chaperone activity; it was proposed that UNC-45 may act as an assemblase for the sarcomere, as a chaperone for folding the myosin head or both (Venolia and Waterston 1990). Chaperone assays suggested that UNC-45 likely acts to fold the myosin head because UNC-45 has endogenous chaperone activity and localizes to the myosin head in vitro (Barral *et al.* 2002). Our results support this hypothesis but also reveal that UNC-45 and NMY-2 remain co-localized at the cortex of the early embryo suggesting that UNC-45 may play a more significant role once myosin has been properly folded. This role may be to refold myosin after the repeated contractile cycles can cause both thermal and osmotic cellular stresses that may lead to misfolding (Johnson *et al.* 2007).

What was unclear from our initial analysis (Kachur *et al.* 2004; section 2) was whether UNC-45 functioned in all NMY-2 based contractile activities including cytokinesis, anterior-posterior polarity establishment and polar body extrusion (Cuenca *et al.* 2003). Using RNA interference to further deplete the maternal contribution of UNC-45, we were able to determine that UNC-45 was indeed required for all processes where NMY-2 was required. This confirmed our previous data that UNC-45 promotes myosin contractility and it also superseded our initial data by suggesting that UNC-45 is the sole chaperone complex that folds the myosin head.

The detailed analysis of maternal UNC-45 indicated that UNC-45 is required outside the body wall muscle implying that Type II non-muscle myosins follow a similar folding cascade to muscle myosins. Additionally, because non-muscle myosins do not form organized sarcomeres. our data confirm that UNC-45 does not function only as an assemblase to form the sarcomere; although our data cannot rule out this additional function. Previous studies into the biochemistry of UNC-45 revealed that it was required with Hsp90 for generalized folding of the myosin head domain (Barral *et al.* 2002). Using the germline as a system to investigate some of the biochemistry of UNC-45 we were able to determine that UNC-45 is specifically required to fold the actin binding domain. When myosin is chemically inhibited from binding actin, myosin dissociates from the actin microfilaments; however, when *unc-45* is depleted myosin remains co-localized with actin. Therefore, our chemical and genetic analysis has refined the biochemical function of UNC-45.

Hermaphrodites that are depleted for UNC-45 either genetically or by RNA interference are sterile. Analysis of the germline revealed that UNC-45 cooperates with NMY-2 during germline cellularization and that NMY-2 provides the sole contractile force controlling membrane invagination in this tissue. Our work represents the first characterization of the mechanics of germline cellularization in *C. elegans* and suggests that NMY-2 is the major motor molecule required for invagination of PGC membranes and for completion of cellularization into oocytes.

Germline cellularization provides an excellent system to study the regulation of myosin because it is easy to manipulate and a diverse array of reporter molecules are available. The primordial germ cells are formed at the entry into meiosis in the distalmost region of the gonad and this syncytium of PGCs is maintained until entry into the proximal arm of the gonad where cellularization completes. NMY-2::GFP strongly localizes along the PGC membranes suggesting that NMY-2 is required for all stages of cellularization. Our identification of two phases of membrane addition suggests that there are two regulatory events controlling myosin activity – lateral growth and apical closure.

With our new data we can refine the myosin folding cascade to suggest that Hsp90 interacts with newly translated myosin (Figure 5.1A). This interaction likely requires

Hsp70 as well as other heat shock complex components and it may function solely to maintain myosin as an unfolded protein but in a state competent for folding. Because Hsp90 does not have sufficient endogenous chaperone activity to fold the myosin head, UNC-45 is hypothesized to help attain the final conformation. However, we have not shown that Hsp90 can localize to myosin without UNC-45 and alternatively, they may be recruited together (Figure 5.1B). Prior to UNC-45 association the actin binding domain of myosin properly folds (Figure 5.1C) and this folding is likely spontaneous. Additionally, in order for myosin to bind actin without UNC-45 activity, the myosin light chains must associate and be phosphorylated to release myosin from its autoinhibitory state. The activities of myosin dimerization, myosin light chain association and actin binding do not require folding assistance from UNC-45 because they can occur spontaneously in an *in vitro* assay (Chow *et al.* 2000). Folding of the ATP binding domain or other unidentified regions does require UNC-45 chaperone activity (Figure 5.1C). We propose that UNC-45 is required to fold the actin binding domain based on two observations, firstly UNC-45 interacts with NMY-2 near or at the C-terminal portion of the head that corresponds with the ATP binding domain. Secondly, if the ATP binding pocket can bind ATP but not hydrolyze it that would allow for actin binding but not release, and hence contraction, during the myosin contractile cycle. One key observation that has been made both in the embryo and in the muscle is that UNC-45 and myosin remain associated after myosin head folding is complete. We suggest that this is to restore folding in myosin heads that have unfolded because of the stress of the contractile cycle (Figure 5.1D).

Myosin function controls processes ranging from cancer metastasis to fertilization and investigating the factors required for myosin function, such as UNC-45, contributes to our understanding of global cellular function. UNC-45 homologs in vertebrate models have found functions for UNC-45 outside the striated muscle (Wohlgemuth *et al.* 2007; Price *et al.* 2002; Bazzaro *et al.* 2007; Chadli *et al.* 2007) our results have also revealed that UNC-45 functions with different myosin subtypes likely with the same chaperone mechanism. Therefore studying the simple system, *C. elegans* and its one *unc-45* gene can provide evidence for potential roles for the general cell homolog of UNC-45, UNC45a, outside the muscle in vertebrate systems, for instance during cytokinesis in higher eukaryotes.

5.1 Future Directions

There are two possible routes for future research: 1) to focus on the biochemical role for UNC-45 and 2) to focus on the cellular mechanisms that require UNC-45.

Although roles for UNC-45 outside the body wall muscle have been identified and an interaction between UNC-45 and NMY-2 has been shown we do not have evidence for UNC-45 chaperone activity on any nematode myosin *in vitro*. In order to fully characterize the function of UNC-45 chaperone assays must be conducted with myosin as a chaperone substrate. Conformation specific antibodies are available from chicken striated muscle myosin that may recognize similar epitopes in unfolded type II muscle myosins from *C. elegans,* therefore this may provide a possible method to study if myosin can achieve a functional conformation with the addition of UNC-45. Because

UNC-45 interacts with non-muscle myosins, NMY-2 may be easier to purify *in vitro* compared with muscle myosins and may provide a possible substrate for biochemical analysis.

New processes that require UNC-45 and NMY-2 were identified including anterior-posterior polarity establishment, cytokinesis, polar body extrusion and germline cellularization. Much remains to be discovered about the role of both UNC-45 and NMY-2 in these processes. For instance, in the embryo UNC-45 and NMY-2 remain co-localized at the cortex; this pattern is interesting because if UNC-45 was simply acting as a chaperone to fold the myosin head, then this function could be accomplished in the cytoplasm without co-localizing. A hypothesis is that NMY-2 may require refolding assistance after any potential misfolding (Figure 5.1). Providing evidence for this may be problematic but developing an UNC-45::CFP reporter strain to look at the dynamics of UNC-45 compared with NMY-2 may begin to address this. If UNC-45::CFP is more dynamic than NMY-2::GFP with its association and dissociation from the cortex then one may suppose that UNC-45 is being recruited as needed to refold myosin. Another possibility is that UNC-45 is not required at the cortex but remains localized to myosin through a domain that does not interfere with myosin function. Techniques such as fluorescence recovery after photobleaching (FRAP) can address the stability of UNC-45 at the cortex as only static immunofluorescent images have been studied thus far.

Germline cellularization in particular has been largely unstudied and can provide an ideal system to study processes of cellularization furrow positioning and myosin regulation. My hypothesis is that there is a spatial gradient of regulators that separate the germline into two distinct regions, the first where lateral membrane growth occurs and the second at the more proximal region where apical closure occurs. In order to confirm this hypothesis the different molecules that can regulate myosin need to be identified. The best way to approach finding myosin regulators during cellularization would be to conduct a screen using the mCherry:PH strain and look for defects in cellularization. Possible candidates have been identified (Table 4.1) and it is feasible to begin with a small scale candidate gene approach using RNA interference. A second approach to looking at myosin regulators is to use chemical inhibitors of some of the candidates, for instance, ML-7 is a known inhibitor of MLCKs therefore redundancy would not be an obstacle to looking at these germlines. The possibility that the gonad could provide a system to study spatial regulation of myosin contractility is unique amongst model organisms and is a potentially successful avenue to pursue.

The major obstacle to developing a model for germline cellularization is that the only identified regulatory myosin light chain for non-muscle myosins (MLC-4) (Shelton *et al.* 1999) is not required for apical closure. This is either because the strain examined is not a true null or that there is, in fact, a second rMLC in the germline. Two other muscle associated regulatory myosin light chains exist in the genome, MLC-1 and MLC-2 (Rushforth *et al.* 1998) but no sterility results when both genes are knocked down by RNAi (Kamath and Ahringer 2003). A fifth myosin light chain, MLC-5 has recently been identified as an essential myosin light chain but this does increase the possibility that, despite a fully sequenced genome, other candidates still exist. Another factor that could be involved during cellularization is *nmy-1*, a second non-muscle Type II myosin that results in partial sterility when knocked down by RNAi (Kamath and Ahringer 2003). Therefore, NMY-1 may have redundancy with *nmy-2*; this can be investigated by analyzing double mutants or double RNAi knockdowns of both NMY-1 and NMY-2 to look for synergistic effects that suggest a functional redundancy.

The mechanics of germ cell formation and the regulation of myosin contraction provides an important avenue of research to pursue and represents the first analysis of germ cell cellularization in the *C. elegans* gonad.

5.2 Conclusions

UNC-45 has been found to be an essential factor in modulating Type II myosin function both within and outside of the body wall muscle in *C. elegans*. Additionally, homologs of UNC-45 are important for acting as a chaperone for the myosin head domain to ensure that contractile processes occur in many different cell types. Studying UNC-45 in the nematode provides an ideal system to study the function of both muscle and non-muscle specific chaperone functions. I have shown that UNC-45 is required outside the body wall muscle for essential processes of cell division, polarity establishment and germ cell formation. These contributions provide exciting avenues of research for UNC-45 both in the nematode model as well as for the two homologs in vertebrate systems.

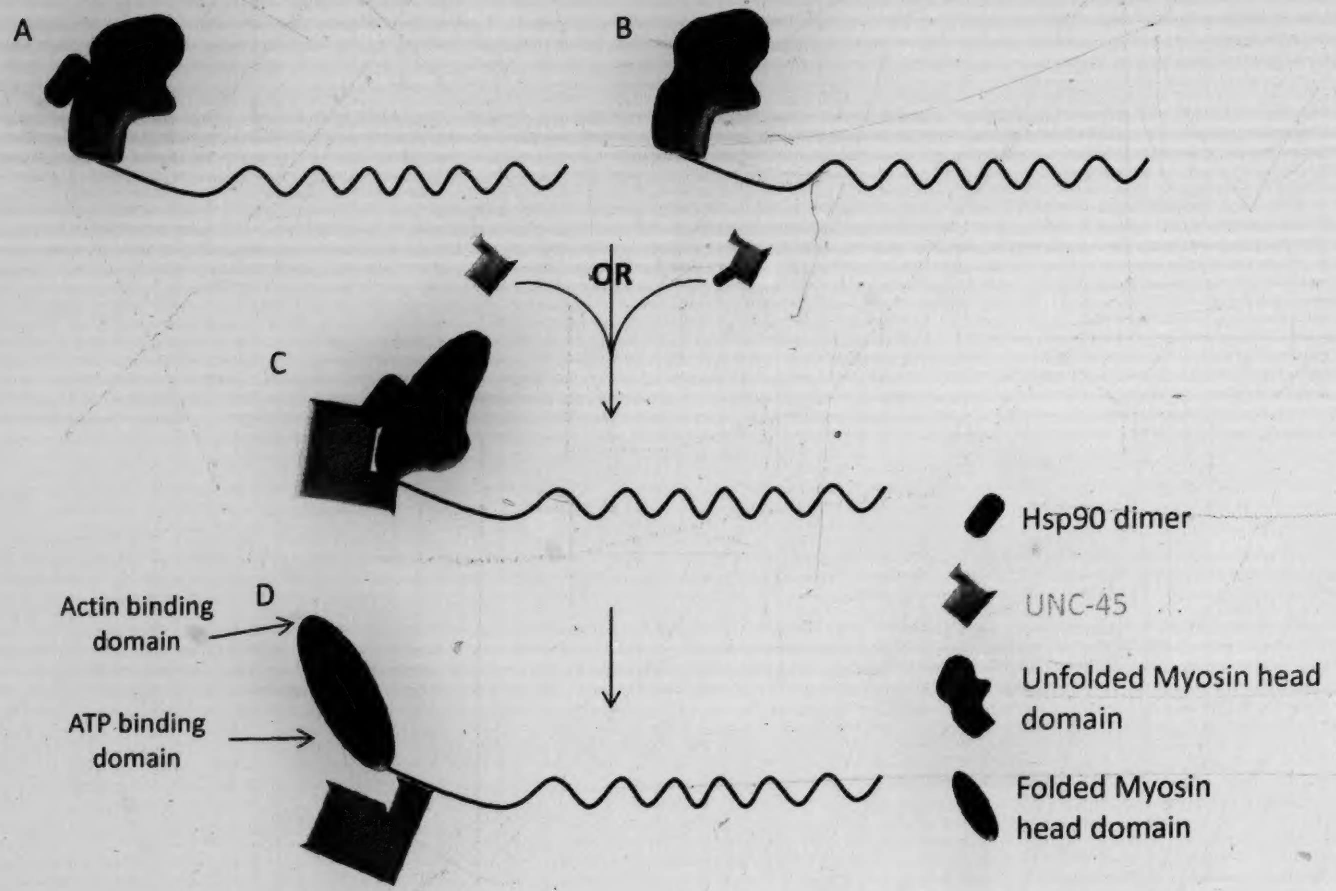
A
B
OR
C
D
Actin binding domain
ATP binding domain
Hsp90 dimer
UNC-45
Unfolded Myosin head domain
Folded Myosin head domain

Figure 5.1. Revised model for UNC-45 function. A) Hsp90 chaperone associates with the unfolded myosin head. B) UNC-45 and Hsp90 may be recruited to myosin as a complex and not in a stepwise manner. C) UNC-45 associates with the neck region of myosin and promotes folding of the myosin head. Myosin can still bind actin without UNC-45 activity suggesting that UNC-45 may only be required late in the head folding cascade to fold a subsection of the head, for example, the ATP binding domain. **D)** UNC-45 co-localizes with NMY-2 suggesting UNC-45 may be required to refold myosin after contraction-induced misfolding.

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